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**(57) Abstract**

The invention relates to novel xylanase activity (XA) proteins and nucleic acids. The invention further relates to the use of the XA proteins in the process of pulp bleaching.

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## NOVEL THERMOSTABLE ALKALIPHILIC XYLANASE

This application is a continuing application of U.S.S.N. 60/133,714, filed May 12, 1999.

## FIELD OF THE INVENTION

The invention relates to xylanase activity (XA) proteins and nucleic acids. The invention further relates to the use of the XA proteins in the bleaching process of pulp and in the food and animal feed industry.

## BACKGROUND OF THE INVENTION

Glycosyl hydrolase enzymes have been classified into more than 60 families that include xylanases (Xyn), cellulases, mannanases, amylases, beta-glucanases, and other carbohydrases [Henrissat, Biochem. J. 280:309-316 (1991); Henrissat and Bairoch, Biochem. J. 293:781-788 (1993); Henrissat and Bairoch, Biochem. J. 316:695-696 (1996); Davies and Henrissat, Structure 3:853-859 (1995); Coutinho and Henrissat, in Genetics, Biochemistry and Ecology of Cellulose Degradation, eds. Ohmiya et al., Uni Publishers Co., Tokyo, pp 15-23 (1999)]. These enzymes are classified based on amino acid sequence, the three dimensional structure and the geometry of the catalytic site [Gilkes et al., Microbiol. Reviews 55:303-315 (1991)]. Xylanases are produced by many organisms of bacterial and fungal origin [*Enzymes for Pulp and Paper Processing*, eds. Jeffries and Viikari; ACS Symposium Series Vol. 655, American Chemical Society, Washington, D.C. (1996); and *Xylans and Xylanases*, eds. Visser et al., Progress in Biotechnology Vol. 7; Amsterdam-London-New York-Tokyo (1992)] and are used to hydrolyze the polysaccharide xylan, which is a major component of the plant cell walls [*Hemicellulose and Hemicellulases*, eds. Coughlan & Hazelwood; Portland Press Ltd, London-Chapel Hill, (1993)].

The endo-beta-1,4-xylanases (EC 3.2.1.8) belong either to the family 10 xylanases, formerly known as F, or to the family 11 xylanases, also known as G. The family 10 have an ( $\alpha/\beta$ )<sub>8</sub> barrel fold [Dominguez et al., Nat. Struc. Biol. 2:29-35 (1995)], whereas the family 11 xylanases are mostly  $\beta$ -sheet and the overall structure resembles that of a right hand [Torronen et al., EMBO J. 13:2493-2501 (1994)]. The *Bacillus circulans* xylanase belongs to the family 11.

Family 11 xylanases have been reported from various microorganism (bacteria, yeast and fungi), including *Aspergillus awamori* var. *kawachi* xyn A [Ito, Swiss prot. Entry P48824]; *Aspergillus niger* Xyn A [Krengel and Dijkstra, J. Mol. Biol. 263(1):70-78 (1996); PDB entry 1ukr]; *Aspergillus kawachii* Xyn C [Ito et al., Biosci. Biotechnol. Biochem. 56(8):1338-1340 (1992)]; *Aspergillus tubigenensis* Xyn A [de Graaff et al., Mol. Microbiol. 12(3):479-490 (1994)]; *Bacillus circulans* Xyn A [Yang et al., Nucl. Acids. Res., 16:7187 (1988)]; *Bacillus pumilus* Xyn A [Fukusaki et al., FEBS Lett. 171:197-201 (1984)]; *Bacillus subtilis* Xyn A [Paice et al., Arch. Microbiol. 144:201-202 (1986)]; *Bacillus* sp. strain 41M-1 [Ryuichiro et al., Nucl. Acids Symp. Series 31:235-236 (1994)]; *Cellulomonas fimi* Xyn D; *Chainia* spp. Xyn; *Clostridium acetobutylicum* Xyn B [Zappe et al., Nucl. Acids Res. 18(8):2179 (1990)]; *Clostridium stercorarium*; Xyn A [Sakka et al., Biosci. Biotechnol. Biochem. 57(2):273-277 (1993)]; *Cochliobolus carbonum* [Apel et al., Mol. Plant Microbe Interact. 6(4):467-473 (1993)]; *Fibrobacter succinogenes* Xyn C [Paradis et al., J. Bacteriol. 175(23):7666-7672 (1993)]; *Neocallimastix patriciarum* Xyn A [Gilbert et al., Mol. Microbiol. 6(15):2065-2072 (1992)]; *Nocardiopsis dassonvillei* Xyn II; *Paecilomyces varotii* [J. Mol. Biol. 243(4):806-808 (1994); PDB entry 1PVX]; *Ruminococcus flavefaciens* Xyn A Zhang and Flint, Mol. Microbiol. 6(8):1013-1023 (1992)]; *Schizophyllum commune* Xyn [Yaguchi et al., in *Xylans and Xylanases*, eds. Visser et al., Progress in Biotechnology Vol. 7; pp 149-154, Amsterdam-London-New York-Tokyo (1992)]; *Streptomyces lividans* Xyn B [Shareck et al., Gene 107(1):75-82 (1991)]; *Streptomyces lividans* Xyn C [Shareck et al., Gene 107(1):75-82 (1991)]; *Streptomyces* sp. No. 36a Xyn [Nagashima et al., Trends Actinomycetologia 91-96 (1989)]; *Streptomyces thermoviolaceus* Xyn II; *Thermomonospora fusca* Xyn A; *Thermomyces lanuginosus* [Gruber et al., Biochemistry 37(39):13475-13485 (1998)]; *Trichoderma harzianum* Xyn [Campbell et al., PDB entry 1XND]; *Trichoderma reesei* Xyn I [Torronen and Rouvinen, Biochemistry 34:847 (1995); PDB entry 1XYN]; *Trichoderma reesei* Xyn II [Torronen et al., EMBO J. 13(11):2493-2501 (1994); PDB entry 1ENX]; *Trichoderma viride* Xyn [Yaguchi, GenBank accession #A44594; (gi:627019)].

In recent years, xylanases have become more and more used in the pulp and paper industry in a process called kraft pulp bleaching [Enzymes for Pulp and Paper Processing, eds. Jeffries and Viikari; ACS Symposium Series Vol. 655, American Chemical Society, Washington, D.C. (1996)]. These enzymes are added to the pulp before the pulp is bleached, to enhance the bleaching process and to remove a portion of the xylan in the pulp [Paice and Jurasek, J. Wood Chem. Tech. 4(2):187-198 (1984)]. This enzymatic pre-treatment allows the subsequent bleaching chemicals, including chlorine, chlorine dioxide, hydrogen peroxide, oxygen, ozone, and sodium hydroxide, to bleach the pulp more efficiently than in the absence of xylanase treatment. The enhanced efficiency of bleaching has allowed mills to reduce the amount of chlorine-based chemicals used, thereby decreasing the amount of toxic by-products, which are environmental pollutants. In addition, less bleaching chemicals are used, lowering the chemical costs.

The Family 11 xylanases have several advantages over other xylanases in pulp bleaching applications. Most of the Family 11 xylanases are smaller than xylanases in other families. The small size relative to other xylanases is probably beneficial in penetrating the pulp fibers to release xylan from the pulp and enhance the bleaching. The Family 11 xylanases are also "pure" xylanases in terms of their catalytic activity. Unlike the xylanase enzymes in other families, these enzymes hydrolyze only xylan and do not hydrolyze cellulose. Cellulose hydrolysis damages the pulp and is unacceptable in a commercial mill.

In spite of the advantages of Family 11 xylanases in pulp bleaching, these enzymes have significant drawbacks. The range of temperature and pH that the enzymes exhibit activity on pulp are 45°C to 55°C and pH 5.0 to 7.5. A small proportion of mills have operated historically within these ranges. However, the step in the process where xylanase is applied is after a hot alkali treatment, so that the pulp is very basic and hot, typically having a temperature of 60°C to 70°C and a pH of 10 to 12. Both of these conditions are sub-optimal for xylanase enzymatic activity. For example, the *Bacillus circulans* wild type xylanase has a temperature optimum of 55°C and a pH optimum of 5.5. In some mills the adjustment of temperature and pH are acceptable and routine, albeit energy intensive and costly. In many mills achieving the desired treatment conditions causes severe problems. Therefore, the intrinsic properties of the enzyme, such as thermostability and activity at elevated pH are critical parameters for their use in the bio-bleaching processes.

Among naturally occurring xylanases, thermostable enzymes have been isolated from thermophilic microbes, such as *Caldocellum saccharolyticum*, *Thermatoga maritima* and *Thermatoga sp.* strain FjSS3-B.1, all of which grow at 80°C to 100°C [Luthi et al. Appl. Environ. Microbiol. 56:2677-2683 (1990); Winterhalter and Liebl, Appl. Environ. Microbiol. 61:1810-1815 (1995); Simpson et al., Biochem. J. 277:413-417 (1991)]. However, all are relatively large in size with high molecular mass of 35-120 kDa (320-1100 residues) and as such, their penetration into the pulp fibers might be limited. Some of these xylanases (e.g., *C. saccharolyticum* xylanase A) belong to families other than Family 11, and have both xylanase and cellulase activities (Luthi, et al., supra). Such cellulase activity is undesirable for pulp bleaching. Furthermore, hyperthermostable xylanases which function normally at extremely high temperatures have low activity at the comparatively lower temperatures for pulp bleaching.

It is well known in the art that xylanase also has uses in non-pulp applications. For example, xylanases have been reported to be useful in clarifying juice and wine [Zeikus et al., ACS Symp. Ser. 460:36-51 (1991); Beily, ACS Symp. Ser. 460:408-416 (1991); Woodward, Top Enzyme Ferment. Biotechnol.

8:9-30 (1984)]; extracting coffee, plant oils and starch [Beily, supra; Woodward supra; McCleary, Int. J. Biol. Macromol. 8:349-354 (1986)]; for the production of food thickeners (Zeikus et al., supra); altering texture in bakery products, e.g., improving the quality of dough, help bread rise and processing of wheat and corn for starch production (Maat et al. In *Xylans and xylanases*, eds. Visser et al., Elsevier Sci pub., Amsterdam. ISBN 0-444-894-772 (1992); McCleary, supra; Krishnarau et al., J. Food Sci. 59:1251-1254 (1994); US Patent No. 5,306,633); for use as animal food additives to aid in the digestibility of feedstuffs; and in the washing of super precision devices and semiconductors [Takayuki et al., U.S. Pat. No. 5,078,802). Several of these application could benefit from a thermostable xylanase, for example, food processing at elevated temperatures.

The active site of the *Bacillus circulans* xylanase is a wide cleft with two catalytic glutamates, E78 and E172, on either side and several aromatic tryptophan and tyrosine residues which act as binding sites for the substrate. The enzymatic mechanism consists of a nucleophilic attack of E78 on the 1,4-glycoside bond that is followed by a proton transfer from the acid/base catalyst E172 and a subsequent attack of a solvent water molecule where E172 now acts as a base. The enzymatic reaction results in retention of the configuration at the anomeric carbon [McCarter and Withers, Curr. Opin. Struc. Biol. 4:885-892 (1994)].

To this end, variants of xylanase (Xyn) sequences, applications and production procedures are known; see for example U.S. Patent Nos. 5,405,769; 5,736,384; 5,759,840; Arase et al. [FEB Lett. 316(2):123-7 (1993)]; Wakarchuk et al. [Protein Sci. 3(3):467-75 (1994); Protein Eng. 7(11):1379-86 (1994)]; and references cited therein.

Recently, the crystal structures of recombinant *Bacillus circulans* xylanase [PDB entry 1XNB; Campbell et al., in Suominen and Geinikainen, eds. *Proceedings of the second TRICEL symposium on Trichoderma reesei cellulases and other hydrolases*, Espoo, Finland, Helsinki: Foundation for Biotechnological and Industrial Fermentation Research, pp 63-77 (1993)]; expressly incorporated by reference) have been solved. In addition, structures for xylanases from *Aspergillus niger* Xyn A [Krengel and Dijkstra, J. Mol. Biol. 263(1):70-78 (1996); PDB entry 1ukr]; *Paecilomyces varotii* [J. Mol. Biol. 243(4):806-808 (1994); PDB entry 1PVX]; *Trichoderma harzianum* [Campbell et al., PDB entry 1XND]; *Trichoderma reesei* [Torronene and Rouvinen, Biochemistry 34:847 (1995); PDB entry 1XYN]; *Trichoderma reesei* [Torronene et al., EMBO J. 13(11):2493-2501 (1994); PDB entry 1ENX], all of which are expressly incorporated by reference. The three-dimensional structure of *Bacillus circulans* xylanase is composed of three beta-sheets and one alpha-helix. The first two beta-sheets (I and II) are roughly parallel, while the third one (sheet III) is at about a 90 degree angle to sheet II. Sheets I and II are each composed of five strands, while sheet III contains six strands. The alpha-helix lies across the back of sheet III and the last two strands of sheet III fold over one edge of the alpha-helix. The

active site lies in the cleft between sheets II and III (PDB entry 1XNB; US Patent No. 5,405,769, herewith expressly incorporated as reference).

When carrying out protein engineering to modify protein properties, usually one had to select from the following options: (i) site-specific mutagenesis and (ii) random mutagenesis of the nucleic acid encoding the protein, or (iii) post-translational chemical modifications. No matter which method of protein engineering is used, a key aspect is determining which amino acids to modify, because few choices will improve the properties of the protein. The available crystal structure of xylanase allows a completely different approach by using computational protein design and the generation of more stable proteins or protein variants with an altered activity. Several groups have applied and experimentally tested systematic, quantitative methods to protein design with the goal of developing general design algorithms (Hellings et al., J. Mol. Biol. 222: 763-785 (1991); Hurley et al., J. Mol. Biol. 224:1143-1154 (1992); Desjarlais et al., Protein Science 4:2006-2018 (1995); Harbury et al., Proc. Natl. Acad. Sci. U.S.A. 92:8408-8412 (1995); Klemba et al., Nat. Struc. Biol. 2:368-373 (1995); Nautiyal et al., Biochemistry 34:11645-11651 (1995); Betzo et al., Biochemistry 35:6955-6962 (1996); Dahiyat et al., Protein Science 5:895-903 (1996); Dahiyat et al., Science 278:82-87 (1997); Dahiyat et al., J. Mol. Biol. 273:789-96; Dahiyat et al., Protein Sci. 6:1333-1337 (1997); Jones, Protein Science 3:567-574 (1994); Kono, et al., Proteins: Structure, Function and Genetics 19:244-255 (1994)). These algorithms consider the spatial positioning and steric complementarity of side chains by explicitly modeling the atoms of sequences under consideration. In particular, WO98/47089, and U.S.S.N. 09/127,926 describe a system for protein design; both are expressly incorporated by reference.

A need still exists for xylanase enzymes exhibiting both significant thermostability, thermophilicity, alkalophilicity and xylanase activity. It is therefore an object of this invention, to provide novel xylanase activity (XA) proteins that are active at higher pH and temperature ranges as the naturally occurring xylanases. The novel XA proteins may find wider application in the pre-treatment of kraft pulp and other applications.

#### SUMMARY OF THE INVENTION

In accordance with the objects outlined above, the present invention provides non-naturally occurring xylanase activity (XA) proteins (e.g. the proteins are not found in nature) comprising amino acid sequences that are less than about 97% identical to *Bacillus circulans* xylanase. The XA proteins have at least one altered biological property when compared to *Bacillus circulans* xylanase; for example, the XA proteins will be more alkalophilic or more thermophilic or more thermostable or hydrolyze a substrate more efficiently than *Bacillus circulans* xylanase. Thus, the invention provides

XA proteins with amino acid sequences that have at least about 3-5 amino acid substitutions as compared to the *Bacillus circulans* xylanase sequence shown in Figure 1.

In a further aspect, the present invention provides non-naturally occurring XA conformers that have three dimensional backbone structures that substantially correspond to the three dimensional backbone structure of *Bacillus circulans* xylanase. The amino acid sequence of the XA conformer and the amino acid sequence of *Bacillus circulans* xylanase are less than about 97% identical. In one aspect, at least about 90% of the non-identical amino acids are in a core region of the conformer. In other aspects, the conformer have at least about 100% of the non-identical amino acids are in a core region of the conformer.

In an additional aspect, the changes are selected from the amino acid residues at positions selected from positions 7, 26, 28, 30, 39, 53, 58, 63, 64, 65, 67, 79, 80, 83, 84, 85, 88, 96, 98, 100, 102, 103, 105, 109, 110, 118, 128, 129, 130, 132, 136, 142, 144, 147, 148, 149, 150, 152, 156, 158, 160, 167, 168, 171, 176, 180, and 182. In a preferred aspect, the changes are selected from the amino acid residues at positions selected from positions 26, 28, 30, 53, 58, 64, 79, 105, 142, 171, 176, 180, and 182. In one aspect, the changes are selected from the amino acid residues at positions selected from positions 53, 83, 84, 85, 105, 132, 136, 142, 144, and 149. In another aspect, the changes are selected from the amino acid residues at positions selected from positions 79, 96, 98, 100, 102, 103, 105, 109, 128, 130, 132, 144, 147, 148, 149, 150, 152, 156, 158, 160, and 167. In another aspect, the changes are selected from the amino acid residues at positions selected from positions 7, 39, 63, 65, 67, 88, 110, 118, 129, and 168. Preferred embodiments include at least about 3-5 variations.

In a further aspect, the invention provides recombinant nucleic acids encoding the non-naturally occurring XA proteins, expression vectors comprising the recombinant nucleic acids, and host cells comprising the recombinant nucleic acids and expression vectors.

In an additional aspect, the invention provides methods of producing the XA proteins of the invention comprising culturing host cells comprising the recombinant nucleic acids under conditions suitable for expression of the nucleic acids. The proteins may optionally be recovered.

In a further aspect, the invention provides a bleaching agent comprising as an active ingredient an XA protein.

In an additional aspect of the invention, the invention provides a method for bleaching pulp, said method comprising the step of contacting pulp to be bleached with the bleaching agent. The method may further comprise the step of chemical bleaching and /or an alkali extraction before, after or during said step of contacting pulp with said bleaching agent.



## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1A depicts the amino acid sequence of the endo-1,4-beta xylanase precursor (Xylanase; 1,4-beta-D-xylan xylanohydrolase; E.C. 3.2.1.8) as deposited under GenBank accession numbers P09850 and CAA30553 [see also Yang et al., Nucleic Acids Res. 16 (14B):7187 (1988)]. Amino acid residues 1-28 correspond to the signal peptide and amino acid residues 29-213 correspond to the mature protein.

Figure 1B depicts the amino acid sequence of *Bacillus circulans* xylanase as used in the determination of the crystal structure [PDB and GenBank # 1XNB; Campbell et al., in Suominen and Geinikainen, eds., *Proceedings of the second TRICEL symposium on Trichoderma reesei cellulases and other hydrolases*, Espoo, Finland, Helsinki: Foundation for Biotechnological and Industrial Fermentation Research, pp 63-77 (1993)] and secondary structure elements. Secondary structure element legend: H, alpha helix (4-helix); B, residue in isolated beta bridge; E, extended strand, participates in beta ladder; G, 310 helix (3-helix); I, pi helix (5-helix); T, hydrogen bonded turn; S, bend. Amino acid residues 1 to 185 correspond to amino acid residues 29-213 of the amino acid sequence of Figure 1A. The amino acid numbers shown were used as the amino acid numbers in the design of XA proteins.

Figure 1C depicts the complete DNA sequence encoding wild type *Bacillus circulans* xylanase (Yang et al., Nucleic Acids Res. 16 (14B):7187 (1988); GenBank accession number X07723). The encoded sequence consists of the signaling sequence, MFKFKKNFLVGLSAAALMSISLFSATASA, and the 185 amino acids that constitute the actual protein (see Figures 1A and 1B). The DNA sequence of 1349 nucleotides includes this coding sequence and non-translated 5' and 3' sequences. Bases 392 to 1033 encode wild type *Bacillus circulans* xylanase (213 amino acid residues).

Figure 2A depicts the structure of wild type *Bacillus circulans* xylanase (PDB structure 1XNB). The side chains are drawn for those residues that are included in a PDA CORE design.

Figure 2B depicts the structure of wild type *Bacillus circulans* xylanase (PDB structure 1XNB). The side chains are drawn for those residues that are included in a PDA design of the area around the buried polar residue D83.

Figure 2C depicts the structure of wild type *Bacillus circulans* xylanase (PDB structure 1XNB.) The side chains are drawn for those residues that are included in a PDA design of the area around the helix.

Figure 2D depicts the active site of *B. circulans* xylanase based on PDB entry 1XNB. Those positions included in the PDA design of the active site are shown by their side chain representation. In black are wild type residues, (their conformation was allowed to change, but not their amino acid identity). In grey are positions whose conformation and identity were allowed to change (to any amino acid except proline, cysteine and glycine).

Figure 3 depicts the residues of the wild type bacillus circulans xylanase sequence that are selected for the indicated PDA designs as indicated: for the CORE region, the region around D83, the region around the helix, and the region around the active site. The individual sets are described in detail herein.

Figure 4A depicts the mutation pattern of XA protein CORE sequences based on the analysis of the lowest 1000 protein sequences generated by Monte Carlo analysis of XA protein core sequences. The probability table shows only the amino acid residues of positions 26, 28, 30, 36, 38, 51, 53, 55, 58, 62, 64, 66, 68, 70, 72, 77, 79, 81, 105, 107, 130, 142, 144, 146, 153, 169, 171, 173, 176, 178, 180, 182, and 184. The presence of each amino acid residue at a given position is indicated as %. For example, at position 26, the *Bacillus circulans* xylanase amino acid is tyrosine (see Figure 1); in XA proteins, >90% of the top 1000 sequences had phenylalanine at this position, and only <1% of the sequences had tyrosine.

Figure 4B depicts a preferred XA protein sequence based on the PDA analysis of Example 2. Amino acid residues different from the *Bacillus circulans* xylanase (see Figure 1) are shown in bold and are underlined.

Figure 5A depicts the mutation pattern of XA protein sequences analyzed at the 'Region around D83'. The analysis is based on the calculation parameters described in Example 3(a) and on the analysis of the lowest 1000 protein sequences generated by Monte Carlo analysis of XA protein 'Region around D83' sequences. The probability table shows only the amino acid residues of positions 53, 66, 67, 68, 81, 82, 83, 84, 85, 101, 105, 132, 136, 138, 142, 144, 149, and 169. The occurrence of each amino acid residue at a given position is indicated as %. For example, at position 53, the *Bacillus circulans* xylanase amino acid is tyrosine (see Figure 1); in XA proteins, all of the top 1000 sequences had phenylalanine at this position.

Figure 5B depicts a preferred XA protein sequence based on the PDA analysis of Example 3(a). Amino acid residues different from the *Bacillus circulans* xylanase (see Figure 1) are shown in bold and are underlined.

Figure 6A depicts the mutation pattern of XA protein sequences analyzed at the 'Region around D83'. This analysis is based on the calculation parameters described in Example 3(b) and on the analysis of the lowest 1000 protein sequences generated by Monte Carlo analysis of XA protein 'Region around D83' sequences. See legend of Figure 5A for further details.

- 5 Figure 6B depicts a preferred XA protein sequence based on the PDA analysis of Example 3(b). Amino acid residues different from the *Bacillus circulans* xylanase (see Figure 1) are shown in bold and are underlined.

- 10 Figure 7A depicts the mutation pattern of XA protein sequences analyzed at the 'Region around D83'. The analysis is based on the calculation parameters described in Example 3(c) and on the analysis of the lowest 1000 protein sequences generated by Monte Carlo analysis of XA protein 'Region around D83' sequences. The probability table shows only the amino acid residues of positions 53, 66, 68, 81, 83, 84, 101, 105, 132, 136, 138, 142, 144, 149, and 169. The occurrence of each amino acid residue at a given position is indicated as %. For example, at position 136, the *Bacillus circulans* xylanase amino acid is arginine (see Figure 1); in XA proteins, of the top 1000 sequences >90% had methionine.  
15 at this position and some XA proteins had lysine, leucine, glutamic acid and phenylalanine. None of the XA proteins had arginine at this position.

Figure 7B depicts a preferred XA protein sequence based on the PDA analysis of Example 3(c). Amino acid residues different from the *Bacillus circulans* xylanase (see Figure 1) are shown in bold and are underlined.

- 20 Figure 8A depicts the mutation pattern of XA protein sequences analyzed at the 'Region around D83'. This analysis is based on the calculation parameters described in Example 3(d) and on the analysis of the lowest 1000 protein sequences generated by Monte Carlo analysis of XA protein 'Region around D83' sequences. See legend of Figure 7A for further details.

- 25 Figure 8B depicts a preferred XA protein sequence based on the PDA analysis of Example 3(d). Amino acid residues different from the *Bacillus circulans* xylanase (see Figure 1) are shown in bold and are underlined.

- 30 Figure 9A depicts the mutation pattern of XA protein sequences analyzed at the 'Region around D83'. This analysis is based on the calculation parameters described in Example 3(e) and on the analysis of the lowest 1000 protein sequences generated by Monte Carlo analysis of XA protein 'Region around D83' sequences. See legend of Figure 5A for further details.

Figure 9B depicts a preferred XA protein sequence based on the PDA analysis of Example 3(e). Amino acid residues different from the *Bacillus circulans* xylanase (see Figure 1) are shown in bold and are underlined.

5 Figure 10A depicts the mutation pattern of XA protein sequences analyzed at the 'Region around D83'. This analysis is based on the calculation parameters described in Example 3(f) and on the analysis of the lowest 1000 protein sequences generated by Monte Carlo analysis of XA protein 'Region around D83' sequences. See legend of Figure 5A for further details.

10 Figure 10B depicts a preferred XA protein sequence based on the PDA analysis of Example 3(f). Amino acid residues different from the *Bacillus circulans* xylanase (see Figure 1) are shown in bold and are underlined.

15 Figure 11A depicts the mutation pattern of XA protein sequences analyzed at the 'Helix Region'. The analysis is based on the calculation parameters described in Example 4(a) and on the analysis of the lowest 1000 protein sequences generated by Monte Carlo analysis of XA protein 'Helix Region' sequences. The probability table shows only the amino acid residues of positions 70, 72, 77, 79, 81, 95, 96, 98, 100, 101, 102, 103, 105, 107, 109, 128, 130, 132, 144, 146, 147, 148, 149, 150, 152, 153, 156, 157, 158, 160, 161, 164, and 167. The occurrence of each amino acid residue at a given position is indicated as %. For example, at position 79, the *Bacillus circulans* xylanase amino acid is tyrosine (see Figure 1); in XA proteins, all of the top 1000 sequences had phenylalanine at this position.

20 Figure 11B depicts a preferred XA protein sequence based on the PDA analysis of Example 4(a). Amino acid residues different from the *Bacillus circulans* xylanase (see Figure 1) are shown in bold and are underlined.

25 Figure 12A depicts the mutation pattern of XA protein sequences analyzed at the 'Helix Region'. This analysis is based on the calculation parameters described in Example 4(b) and on the analysis of the lowest 1000 protein sequences generated by Monte Carlo analysis of XA protein 'Helix Region' sequences. Asterisks indicate wild type residues that are included in the PDA calculation but cannot change their identity. See legend of Figure 11A for further details.

Figure 12B depicts a preferred XA protein sequence based on the PDA analysis of Example 4(b). Amino acid residues different from the *Bacillus circulans* xylanase (see Figure 1) are shown in bold and are underlined.

30 Figure 13A depicts the mutation pattern of XA protein sequences analyzed at the 'Helix Region'. This analysis is based on the calculation parameters described in Example 4(c) and on the analysis of the

lowest 1000 protein sequences generated by Monte Carlo analysis of XA protein 'Helix Region' sequences. See legend of Figure 10A for further details.

Figure 13B depicts a preferred XA protein sequence based on the PDA analysis of Example 4(c). Amino acid residues different from the *Bacillus circulans* xylanase (see Figure 1) are shown in bold and are underlined.

Figure 14A depicts the mutation pattern of XA protein sequences analyzed at the 'Active Site Region'. The analysis is based on the calculation parameters described in Example 5(a) and on the analysis of the lowest 10,000 protein sequences generated by Monte Carlo analysis of XA protein 'Active Site Region' sequences. The probability table shows only the amino acid residues of positions 5, 7, 11, 37, 39, 63, 65, 67, 71, 80, 82, 88, 110, 115, 118, 125, 129, 168, and 170. The occurrence of each amino acid residue at a given position is indicated as %. Only those amino acids with a probability greater than 1% are shown. For example, at position 110, the *Bacillus circulans* xylanase amino acid is threonine (see Figure 1); in XA proteins, within the top 1000 sequences aspartic acid is the preferred amino acid at this position (99.9%).

Figure 14B depicts a preferred XA protein sequence based on the PDA analysis of Example 5(a). Amino acid residues different from the *Bacillus circulans* xylanase (see Figure 1) are shown in bold and are underlined.

Figure 15A depicts the mutation pattern of XA protein sequences analyzed at the 'Active Site Region with Substrate'. This analysis is based on the calculation parameters described in Example 5(b) and on the analysis of the lowest 10,000 protein sequences generated by Monte Carlo analysis of XA protein 'Active Site Region with Substrate' sequences. See legend of Figure 14A for further details.

Figure 15B depicts a preferred XA protein sequence based on the PDA analysis of Example 5(b). Amino acid residues different from the *Bacillus circulans* xylanase (see Figure 1) are shown in bold and are underlined.

Figure 16A depicts the amino acid sequence of *Bacillus subtilis* xylanase.

Figure 16B depicts the amino acid sequence of *Bacillus pumilus* xylanase.

Figure 16C depicts the amino acid sequence of *Streptomyces lividans* xylanase B.

Figure 16D depicts the amino acid sequence of *Streptomyces lividans* xylanase C.

Figure 16E depicts the amino acid sequence of *Clostridium acetobutylicum* xylanase.

Figure 16F depicts the amino acid sequence of *Schizophyllum commune* xylanase.

Figure 16G depicts the amino acid sequence of *Trichoderma viride* xylanase.

Figure 16H depicts the amino acid sequence of *Trichoderma harzianum* xylanase.

5 Figure 16I depicts the amino acid sequence of *Trichoderma reesei* xyn I xylanase.

Figure 16J depicts the amino acid sequence of *Trichoderma reesei* xyn II xylanase.

Figure 16K depicts the amino acid sequence of *Paecilomyces variotii* xylanase.

Figure 16L depicts the amino acid sequence of *Thermomyces lanuginosus* xylanase.

Figure 16M depicts the amino acid sequence of *Aspergillus niger* xylanase.

10 Figure 16N depicts the amino acid sequence of *Aspergillus awamori* var. *kawachi* xylanase A.

Figure 16O depicts the amino acid sequence of *Aspergillus awamori* var. *kawachi* xylanase.

Figure 16P depicts the amino acid sequence of *Neocalimastix patriciarum* xylanase.

Figure 16Q depicts the amino acid sequence of *Cochliobolus carbonum* xylanase.

Figure 16R depicts the amino acid sequence of *Clostridium stercorarium* xylanase.

15 Figure 16S depicts the amino acid sequence of *Ruminococcus flavefaciens* xylanase.

Figure 16T depicts the amino acid sequence of *Fibrobacter succinogenes* xylanase.

Figure 16U depicts the amino acid sequence of *Aspergillus tubigenensis* xylanase.

Figure 16V depicts the amino acid sequence of *Bacillus* sp. strain 41M-1 xylanase.

20 Figure 17 depicts the synthesis of a full-length gene and all possible mutations by PCR. Overlapping oligonucleotides corresponding to the full-length gene (black bar, Step 1) and comprising one or more

desired mutations are synthesized, heated and annealed. Addition of DNA polymerase to the annealed oligonucleotides results in the 5' to 3' synthesis of DNA (Step 2) to produce longer DNA fragments (Step 3). Repeated cycles of heating, annealing, and DNA synthesis (Step 4) result in the production of longer DNA, including some full-length molecules. These can be selected by a second round of PCR using primers (indicated by arrows) corresponding to the end of the full-length gene (Step 5).

Figure 18 depicts a preferred scheme for synthesizing a XA protein library of the invention. The wild type gene, or any starting gene, such as the gene for the global minima gene, can be used. Oligonucleotides comprising sequences that encode different amino acids at the different variant positions (indicated in the Figure by box 1, box 2, and box 3) can be used during PCR. Those primers can be used in combination with standard primers. This generally requires fewer oligonucleotides and can result in fewer errors.

Figures 19A and 19B depict an overlapping extension method. At the top of Figure 19A is the template DNA showing the locations of the regions to be mutated (black boxes) and the binding sites of the relevant primers (arrows). The primers R1 and R2 represent a pool of primers, each containing a different mutation; as described herein, this may be done using different ratios of primers if desired. The variant position is flanked by regions of homology sufficient to get hybridization. Thus, as shown in this example, oligos R1 and F2 comprise a region of homology and so do oligos R2 and F3. In this example, three separate PCR reactions are done for step 1. The first reaction contains the template plus oligos F1 and R1. The second reaction contains template plus oligos F2 and R2, and the third contains the template and oligos F3 and R3. The reaction products are shown. In Step 2, the products from Step 1 tube 1 and Step 1 tube 2 are taken. After purification away from the primers, these are added to a fresh PCR reaction together with F1 and R4. During the denaturation phase of the PCR, the overlapping regions anneal and the second strand is synthesized. The product is then amplified by the outside primers, F1 and R4. In Step 3, the purified product from Step 2 is used in a third PCR reaction, together with the product of Step 1, tube 3 and the primers F1 and R3. The final product corresponds to the full length gene and contains the required mutations. Alternatively, Step 2 and Step 3 can be performed in one PCR reaction.

Figures 20A and 20B depict a ligation of PCR reaction products to synthesize the libraries of the invention. In this technique, the primers also contain an endonuclease restriction site (RE), either generating blunt ends, 5' overhanging ends or 3' overhanging ends. We set up three separate PCR reactions for Step 1. The first reaction contains the template plus oligos F1 and R1. The second reaction contains template plus oligos F2 and R2, and the third contains the template and oligos F3 and R3. The reaction products are shown. In Step 2, the products of Step 1 are purified and then digested with the appropriate restriction endonuclease. The digestion products from Step 2, tube 1 and

Step 2, tube 2 are ligated together with DNA ligase (Step 3). The products are then amplified in Step 4 using oligos F1 and R4. The whole process is then repeated by digesting the amplified products, ligating them to the digested products of Step 2, tube 3, and then amplifying the final product using oligos F1 and R3. It would also be possible to ligate all three PCR products from Step 1 together in one reaction, providing the two restriction sites (RE1 and RE2) were different.

Figure 21 depicts blunt end ligation of PCR products. In this technique, oligos such as F2 and R1 or R2 and F3 do not overlap, but they abut. Again three separate PCR reactions are performed. The products from tube 1 and tube 2 (see Figure 20A, Step 1) are ligated, and then amplified with outside primers F1 and R4. This product is then ligated with the product from Step 1, tube 3. The final products are then amplified with primers F1 and R3.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to novel proteins and nucleic acids possessing xylanase activity (sometimes referred to herein as "XA proteins" and "XA nucleic acids"). The proteins are generated using a system previously described in WO98/47089 and U.S.S.Nos. 09/058,459, 09/127,926, 60/104,612, 60/158,700, 09/419,351, 60/181,630, 60/186,904, 60/132,475, 60/133,714, U.S patent application, entitled *Protein Design Automation For Protein Libraries* (Filed: April 14, 2000; Inventor: Bassil Dahiyat), and PCT US98/07254, all of which are expressly incorporated by reference in their entirety, that is a computational modeling system that allows the generation of extremely stable proteins without necessarily disturbing the biological functions of the protein itself. In this way, novel XA proteins and nucleic acids are generated, that can have a plurality of mutations in comparison to the wild-type enzyme yet retain significant activity.

Generally, there are a variety of computational methods that can be used to generate the XA proteins of the invention. In a preferred embodiment, sequence based methods are used. Alternatively, structure based methods, such as PDA, described in detail below, are used.

Similarly, molecular dynamics calculations can be used to computationally screen sequences by individually calculating mutant sequence scores and compiling a rank ordered list.

In a preferred embodiment, residue pair potentials can be used to score sequences (Miyazawa et al., *Macromolecules* 18(3):534-552 (1985), expressly incorporated by reference) during computational screening.



In a preferred embodiment, sequence profile scores (Bowie et al., Science 253(5016):164-70 (1991), incorporated by reference) and/or potentials of mean force (Hendlich et al., J. Mol. Biol. 216(1):167-180 (1990), also incorporated by reference) can also be calculated to score sequences. These methods assess the match between a sequence and a 3D protein structure and hence can act to screen for fidelity to the protein structure. By using different scoring functions to rank sequences, different regions of sequence space can be sampled in the computational screen.

Furthermore, scoring functions can be used to screen for sequences that would create metal or co-factor binding sites in the protein (Hellinga, Fold Des. 3(1):R1-8 (1998), hereby expressly incorporated by reference). Similarly, scoring functions can be used to screen for sequences that would create disulfide bonds in the protein. These potentials attempt to specifically modify a protein structure to introduce a new structural motif.

In a preferred embodiment, sequence and/or structural alignment programs can be used to generate the XA proteins of the invention. As is known in the art, there are a number of sequence-based alignment programs; including for example, Smith-Waterman searches, Needleman-Wunsch, Double-Affine Smith-Waterman, frame search, Gribskov/GCG profile search, Gribskov/GCG profile scan, profile frame search, Bucher generalized profiles, Hidden Markov models, Hframe, Double Frame, Blast, Psi-Blast, Clustal, and GeneWise.

As is known in the art, there are a number of sequence alignment methodologies that can be used. For example, sequence homology based alignment methods can be used to create sequence alignments of proteins related to the target structure (Altschul et al., J. Mol. Biol. 215(3):403-410 (1990), Altschul et al., Nucleic Acids Res. 25:3389-3402 (1997), both incorporated by reference). These sequence alignments are then examined to determine the observed sequence variations. These sequence variations are tabulated to define a set of XA proteins.

Sequence based alignments can be used in a variety of ways. For example, a number of related proteins can be aligned, as is known in the art, and the "variable" and "conserved" residues defined; that is, the residues that vary or remain identical between the family members can be defined. These results can be used to generate a probability table, as outlined below. Similarly, these sequence variations can be tabulated and a secondary library defined from them as defined below. Alternatively, the allowed sequence variations can be used to define the amino acids considered at each position during the computational screening. Another variation is to bias the score for amino acids that occur in the sequence alignment, thereby increasing the likelihood that they are found during computational screening but still allowing consideration of other amino acids. This bias would result in a focused library of XA proteins but would not eliminate from consideration amino acids not found in the alignment. In addition, a number of other types of bias may be introduced. For example, diversity may

be forced; that is, a "conserved" residue is chosen and altered to force diversity on the protein and thus sample a greater portion of the sequence space. Alternatively, the positions of high variability between family members (i.e. low conservation) can be randomized, either using all or a subset of amino acids. Similarly, outlier residues, either positional outliers or side chain outliers, may be eliminated.

Similarly, structural alignment of structurally related proteins can be done to generate sequence alignments (Orengo et al., *Structure* 5(8):1093-108 (1997); Holm et al., *Nucleic Acids Res.* 26(1):316-9 (1998), both of which are incorporated by reference). These sequence alignments can then be examined to determine the observed sequence variations. Libraries can be generated by predicting secondary structure from sequence, and then selecting sequences that are compatible with the predicted secondary structure. There are a number of secondary structure prediction methods such as helix-coil transition theory (Munoz and Serrano, *Biopolymers* 41:495, 1997), neural networks, local structure alignment and others (e.g., see in Selbig et al., *Bioinformatics* 15:1039-46, 1999).

Similarly, as outlined above, other computational methods are known, including, but not limited to, sequence profiling [Bowie and Eisenberg, *Science* 253(5016):164-70, (1991)], rotamer library selections [Dahiyat and Mayo, *Protein Sci.* 5(5):895-903 (1996); Dahiyat and Mayo, *Science* 278(5335):82-7 (1997); Desjarlais and Handel, *Protein Science* 4:2006-2018 (1995); Harbury et al, *Proc. Natl. Acad. Sci. U.S.A.* 92(18):8408-8412 (1995); Kono et al., *Proteins: Structure, Function and Genetics* 19:244-255 (1994); Hellinga and Richards, *Proc. Natl. Acad. Sci. U.S.A.* 91:5803-5807 (1994)]; and residue pair potentials [Jones, *Protein Science* 3: 567-574, (1994)]; PROSA [Heindlich et al., *J. Mol. Biol.* 216:167-180 (1990)]; THREADER [Jones et al., *Nature* 358:86-89 (1992)], and other inverse folding methods such as those described by Simons et al. [*Proteins*, 34:535-543, (1999)], Levitt and Gerstein [*Proc. Natl. Acad. Sci. U.S.A.*, 95:5913-5920, (1998)], Godzik and Skolnick [*Proc. Natl. Acad. Sci. U.S.A.*, 89:12098-102, (1992)], Godzik et al. [*J. Mol. Biol.* 227:227-38, (1992)] and two profile methods [Gribskov et al. *Proc. Natl. Acad. Sci. U.S.A.* 84:4355-4358 (1987) and Fischer and Eisenberg, *Protein Sci.* 5:947-955 (1996), Rice and Eisenberg *J. Mol. Biol.* 267:1026-1038(1997)], all of which are expressly incorporated by reference. In addition, other computational methods such as those described by Koehl and Levitt (*J. Mol. Biol.* 293:1161-1181 (1999); *J. Mol. Biol.* 293:1183-1193 (1999); expressly incorporated by reference) can be used to create a protein sequence library which can optionally then be used to generate a smaller secondary library for use in experimental screening for improved properties and function. In addition, there are computational methods based on forcefield calculations such as SCMF that can be used as well for SCMF, see Delarue et al. *Pac. Symp. Biocomput.* 109-21 (1997); Koehl et al., *J. Mol. Biol.* 239:249-75 (1994); Koehl et al., *Nat. Struct. Biol.* 2:163-70 (1995); Koehl et al., *Curr. Opin. Struct. Biol.* 6:222-6 (1996); Koehl et al., *J. Mol. Biol.* 293:1183-93 (1999); Koehl et al., *J. Mol. Biol.* 293:1161-81 (1999); Lee J., *Mol. Biol.* 236:918-39 (1994); and Vasquez *Biopolymers* 36:53-70 (1995); all of which are expressly incorporated by

reference. Other forcefield calculations that can be used to optimize the conformation of a sequence within a computational method, or to generate de novo optimized sequences as outlined herein include, but are not limited to, OPLS-AA [Jorgensen et al., J. Am. Chem. Soc. 118:11225-11236 (1996); Jorgensen, W.L.; BOSS, Version 4.1; Yale University: New Haven, CT (1999)]; OPLS  
5 [Jorgensen et al., J. Am. Chem. Soc. 110:1657ff (1988); Jorgensen et al., J. Am. Chem. Soc. 112:4768ff (1990)]; UNRES (United Residue Forcefield; Liwo et al., Protein Science 2:1697-1714 (1993); Liwo et al., Protein Science 2:1715-1731 (1993); Liwo et al., J. Comp. Chem. 18:849-873 (1997); Liwo et al., J. Comp. Chem. 18:874-884 (1997); Liwo et al., J. Comp. Chem. 19:259-276 (1998); Forcefield for Protein Structure Prediction (Liwo et al., Proc. Natl. Acad. Sci. U.S.A. 96:5482-5485 (1999)];  
10 ECEPP/3 [Liwo et al., J. Protein Chem. 13(4):375-80 (1994)]; AMBER 1.1 force field (Weiner et al., J. Am. Chem. Soc. 106:765-784); AMBER 3.0 force field [U.C. Singh et al., Proc. Natl. Acad. Sci. U.S.A. 82:755-759 (1985)]; CHARMM and CHARMM22 (Brooks et al., J. Comp. Chem. 4:187-217); cvff3.0 [Dauber-Osguthorpe et al., Proteins: Structure, Function and Genetics, 4:31-47 (1988)]; cff91 (Maple et al., J. Comp. Chem. 15:162-182); also, the DISCOVER (cvff and cff91) and AMBER forcefields are  
15 used in the INSIGHT molecular modeling package (Biosym/MSI, San Diego California) and CHARMM is used in the QUANTA molecular modeling package (Biosym/MSI, San Diego California), all of which are expressly incorporated by reference. In fact, as is outlined below, these forcefield methods may be used to generate the secondary library directly; that is, no primary library is generated; rather, these methods can be used to generate a probability table from which the secondary library is directly  
20 generated.

In a preferred embodiment, the computational method used to generate the primary library is Protein Design Automation (PDA), as is described in U.S.S.N.s 60/061,097, 60/043,464, 60/054,678, 09/127,926, 60/104,612, 60/158,700, 09/419,351, 60/181,630, 60/186,904, 60/132,475, 60/133,714, U.S. patent application, entitled *Protein Design Automation For Protein Libraries* (Filed: April 14, 2000; Inventor: Bassil Dahiyat) and PCT US98/07254, all of which are expressly incorporated herein by  
25 reference. Briefly, PDA can be described as follows. A known protein structure is used as the starting point. The residues to be optimized are then identified, which may be the entire sequence or subset(s) thereof. The side chains of any positions to be varied are then removed. The resulting structure consisting of the protein backbone and the remaining sidechains is called the template.  
30 Each variable residue position is then preferably classified as a core residue, a surface residue, or a boundary residue; each classification defines a subset of possible amino acid residues for the position (for example, core residues generally will be selected from the set of hydrophobic residues, surface residues generally will be selected from the hydrophilic residues, and boundary residues may be either). Each amino acid can be represented by a discrete set of all allowed conformers of each side  
35 chain, called rotamers. Thus, to arrive at an optimal sequence for a backbone, all possible sequences of rotamers must be screened, where each backbone position can be occupied either by each amino acid in all its possible rotameric states, or a subset of amino acids, and thus a subset of rotamers.

Two sets of interactions are then calculated for each rotamer at every position: the interaction of the rotamer side chain with all or part of the backbone (the "singles" energy, also called the rotamer/template or rotamer/backbone energy), and the interaction of the rotamer side chain with all other possible rotamers at every other position or a subset of the other positions (the "doubles" energy, also called the rotamer/rotamer energy). The energy of each of these interactions is calculated through the use of a variety of scoring functions, which include the energy of van der Waal's forces, the energy of hydrogen bonding, the energy of secondary structure propensity, the energy of surface area solvation and the electrostatics. Thus, the total energy of each rotamer interaction, both with the backbone and other rotamers, is calculated, and stored in a matrix form.

The discrete nature of rotamer sets allows a simple calculation of the number of rotamer sequences to be tested. A backbone of length  $n$  with  $m$  possible rotamers per position will have  $m^n$  possible rotamer sequences, a number which grows exponentially with sequence length and renders the calculations either unwieldy or impossible in real time. Accordingly, to solve this combinatorial search problem, a "Dead End Elimination" (DEE) calculation is performed. The DEE calculation is based on the fact that if the worst total interaction of a first rotamer is still better than the best total interaction of a second rotamer, then the second rotamer cannot be part of the global optimum solution. Since the energies of all rotamers have already been calculated, the DEE approach only requires sums over the sequence length to test and eliminate rotamers, which speeds up the calculations considerably. DEE can be rerun comparing pairs of rotamers, or combinations of rotamers, which will eventually result in the determination of a single sequence which represents the global optimum energy.

Once the global solution has been found, a Monte Carlo search may be done to generate a rank-ordered list of sequences in the neighborhood of the DEE solution. Starting at the DEE solution, random positions are changed to other rotamers, and the new sequence energy is calculated. If the new sequence meets the criteria for acceptance, it is used as a starting point for another jump. After a predetermined number of jumps, a rank-ordered list of sequences is generated. Monte Carlo searching is a sampling technique to explore sequence space around the global minimum or to find new local minima distant in sequence space. As is more additionally outlined below, there are other sampling techniques that can be used, including Boltzman sampling, genetic algorithm techniques and simulated annealing. In addition, for all the sampling techniques, the kinds of jumps allowed can be altered (e.g. random jumps to random residues, biased jumps (to or away from wild-type, for example), jumps to biased residues (to or away from similar residues, for example), etc.). Similarly, for all the sampling techniques, the acceptance criteria of whether a sampling jump is accepted can be altered.

As outlined in U.S.S.N. 09/127,926, the protein backbone (comprising (for a naturally occurring protein) the nitrogen, the carbonyl carbon, the  $\alpha$ -carbon, and the carbonyl oxygen, along with the direction of

the vector from the  $\alpha$ -carbon to the  $\beta$ -carbon) may be altered prior to the computational analysis, by varying a set of parameters called supersecondary structure parameters.

Once a protein structure backbone is generated (with alterations, as outlined above) and input into the computer, explicit hydrogens are added if not included within the structure (for example, if the structure was generated by X-ray crystallography, hydrogens must be added). After hydrogen addition, energy minimization of the structure is run, to relax the hydrogens as well as the other atoms, bond angles and bond lengths. In a preferred embodiment, this is done by doing a number of steps of conjugate gradient minimization [Mayo et al., J. Phys. Chem. 94:8897 (1990)] of atomic coordinate positions to minimize the Dreiding force field with no electrostatics. Generally from about 10 to about 250 steps is preferred, with about 50 being most preferred.

The protein backbone structure contains at least one variable residue position. As is known in the art, the residues, or amino acids, of proteins are generally sequentially numbered starting with the N-terminus of the protein. Thus a protein having a methionine at its N-terminus is said to have a methionine at residue or amino acid position 1, with the next residues as 2, 3, 4, etc. At each position, the wild type (i.e. naturally occurring) protein may have one of at least 20 amino acids, in any number of rotamers. By "variable residue position" herein is meant an amino acid position of the protein to be designed that is not fixed in the design method as a specific residue or rotamer, generally the wild-type residue or rotamer.

In a preferred embodiment, all of the residue positions of the protein are variable. That is, every amino acid side chain may be altered in the methods of the present invention. This is particularly desirable for smaller proteins, although the present methods allow the design of larger proteins as well. While there is no theoretical limit to the length of the protein which may be designed this way, there is a practical computational limit.

In an alternate preferred embodiment, only some of the residue positions of the protein are variable, and the remainder are "fixed", that is, they are identified in the three dimensional structure as being in a set conformation. In some embodiments, a fixed position is left in its original conformation (which may or may not correlate to a specific rotamer of the rotamer library being used). Alternatively, residues may be fixed as a non-wild type residue; for example, when known site-directed mutagenesis techniques have shown that a particular residue is desirable (for example, to eliminate a proteolytic site or alter the substrate specificity of an enzyme), the residue may be fixed as a particular amino acid. Alternatively, the methods of the present invention may be used to evaluate mutations de novo, as is discussed below. In an alternate preferred embodiment, a fixed position may be "floated"; the amino acid at that position is fixed, but different rotamers of that amino acid are tested. In this embodiment, the variable residues may be at least one, or anywhere from 0.1% to 99.9% of the total

number of residues. Thus, for example, it may be possible to change only a few (or one) residues, or most of the residues, with all possibilities in between.

5 In a preferred embodiment, residues which can be fixed include, but are not limited to, structurally or biologically functional residues; alternatively, biologically functional residues may specifically not be fixed. For example, residues which are known to be important for biological activity, such as the residues which the binding site for a binding partner (ligand/receptor, antigen/antibody, etc.), phosphorylation or glycosylation sites which are crucial to biological function, or structurally important residues, such as disulfide bridges, metal binding sites, critical hydrogen bonding residues, residues critical for backbone conformation such as proline or glycine, residues critical for packing interactions, etc. may all be fixed in their amino acid identity and a single rotamer conformation, or "floated", which only fixes the identity but not the rotamer conformation.

10 Similarly, residues which may be chosen as variable residues may be those that confer undesirable biological attributes, such as susceptibility to proteolytic degradation, dimerization or aggregation sites, glycosylation sites which may lead to immune responses, unwanted binding activity, unwanted allostery, undesirable enzyme activity but with a preservation of binding, etc.

15 In a preferred embodiment, each variable position is classified as either a core, surface or boundary residue position, although in some cases, as explained below, the variable position may be set to glycine to minimize backbone strain. In addition, as outlined herein, residues need not be classified, they can be chosen as variable and any set of amino acids may be used. Any combination of core, surface and boundary positions can be utilized: core, surface and boundary residues; core and surface residues; core and boundary residues, and surface and boundary residues, as well as core residues alone, surface residues alone, or boundary residues alone.

20 The classification of residue positions as core, surface or boundary may be done in several ways, as will be appreciated by those in the art. In a preferred embodiment, the classification is done via a visual scan of the original protein backbone structure, including the side chains, and assigning a classification based on a subjective evaluation of one skilled in the art of protein modeling.

25 Alternatively, a preferred embodiment utilizes an assessment of the orientation of the C $\alpha$ -C $\beta$  vectors relative to a solvent accessible surface computed using only the template C $\alpha$  atoms, as outlined in U.S.S.N.s 60/061,097, 60/043,464, 60/054,678, 09/127,926 60/104,612, 60/158,700, 09/419,351, 60/181630, 60/186,904, 60/132,475, 60/133,714, U.S. patent application, entitled *Protein Design Automation For Protein Libraries* (Filed: April 14, 2000; Inventor: Bassil Dahiyat) and PCT US98/07254. Alternatively, a surface area calculation can be done.

Suitable core and boundary positions for XA proteins are outlined below.

Once each variable position is classified as either core, surface or boundary, a set of amino acid side chains, and thus a set of rotamers, is assigned to each position. That is, the set of possible amino acid side chains that the program will allow to be considered at any particular position is chosen.

Subsequently, once the possible amino acid side chains are chosen, the set of rotamers that will be evaluated at a particular position can be determined. Thus, a core residue will generally be selected from the group of hydrophobic residues consisting of alanine, valine, isoleucine, leucine, phenylalanine, tyrosine, tryptophan, and methionine (in some embodiments, when the  $\alpha$  scaling factor of the van der Waals scoring function, described below, is low, methionine is removed from the set), and the rotamer set for each core position potentially includes rotamers for these eight amino acid side chains (all the rotamers if a backbone independent library is used, and subsets if a rotamer dependent backbone is used). Similarly, surface positions are generally selected from the group of hydrophilic residues consisting of alanine, serine, threonine, aspartic acid, asparagine, glutamine, glutamic acid, arginine, lysine and histidine. The rotamer set for each surface position thus includes rotamers for these ten residues. Finally, boundary positions are generally chosen from alanine, serine, threonine, aspartic acid, asparagine, glutamine, glutamic acid, arginine, lysine histidine, valine, isoleucine, leucine, phenylalanine, tyrosine, tryptophan, and methionine. The rotamer set for each boundary position thus potentially includes every rotamer for these seventeen residues (assuming cysteine, glycine and proline are not used, although they can be). Additionally, in some preferred embodiments, a set of 18 naturally occurring amino acids (all except cysteine and proline, which are known to be particularly disruptive) are used.

Thus, as will be appreciated by those in the art, there is a computational benefit to classifying the residue positions, as it decreases the number of calculations. It should also be noted that there may be situations where the sets of core, boundary and surface residues are altered from those described above; for example, under some circumstances, one or more amino acids is either added or subtracted from the set of allowed amino acids. For example, some proteins which dimerize or multimerize, or have ligand binding sites, may contain hydrophobic surface residues, etc. In addition, residues that do not allow helix "capping" or the favorable interaction with an  $\alpha$ -helix dipole may be subtracted from a set of allowed residues. This modification of amino acid groups is done on a residue by residue basis.

In a preferred embodiment, proline, cysteine and glycine are not included in the list of possible amino acid side chains, and thus the rotamers for these side chains are not used. However, in a preferred embodiment, when the variable residue position has a  $\phi$  angle (that is, the dihedral angle defined by 1) the carbonyl carbon of the preceding amino acid; 2) the nitrogen atom of the current residue; 3) the  $\alpha$ -carbon of the current residue; and 4) the carbonyl carbon of the current residue) greater than  $0^\circ$ , the position is set to glycine to minimize backbone strain.

Once the group of potential rotamers is assigned for each variable residue position, processing proceeds as outlined in U.S.S.N. 09/127,926 and PCT US98/07254. This processing step entails analyzing interactions of the rotamers with each other and with the protein backbone to generate optimized protein sequences. Simplistically, the processing initially comprises the use of a number of scoring functions to calculate energies of interactions of the rotamers, either to the backbone itself or other rotamers. Preferred PDA scoring functions include, but are not limited to, a Van der Waals potential scoring function, a hydrogen bond potential scoring function, an atomic solvation scoring function, a secondary structure propensity scoring function and an electrostatic scoring function. As is further described below, at least one scoring function is used to score each position, although the scoring functions may differ depending on the position classification or other considerations, like favorable interaction with an  $\alpha$ -helix dipole. As outlined below, the total energy which is used in the calculations is the sum of the energy of each scoring function used at a particular position, as is generally shown in Equation 1:

Equation 1

$$E_{\text{total}} = nE_{\text{vdw}} + nE_{\text{as}} + nE_{\text{h-bonding}} + nE_{\text{ss}} + nE_{\text{elec}}$$

In Equation 1, the total energy is the sum of the energy of the van der Waals potential ( $E_{\text{vdw}}$ ), the energy of atomic solvation ( $E_{\text{as}}$ ), the energy of hydrogen bonding ( $E_{\text{h-bonding}}$ ), the energy of secondary structure ( $E_{\text{ss}}$ ) and the energy of electrostatic interaction ( $E_{\text{elec}}$ ). The term  $n$  is either 0 or 1, depending on whether the term is to be considered for the particular residue position.

As outlined in U.S.S.N.s 60/061,097, 60/043,464, 60/054,678, 09/127,926, 60/104,612, 60/158,700, 09/419,351, 60/181630, 60/186,904, 60/132,475, 60/133,714, U.S patent application, entitled *Protein Design Automation For Protein Libraries* (Filed: April 14, 2000; Inventor: Bassil Dahiyat) and PCT US98/07254, any combination of these scoring functions, either alone or in combination, may be used. Once the scoring functions to be used are identified for each variable position, the preferred first step in the computational analysis comprises the determination of the interaction of each possible rotamer with all or part of the remainder of the protein. That is, the energy of interaction, as measured by one or more of the scoring functions, of each possible rotamer at each variable residue position with either the backbone or other rotamers, is calculated. In a preferred embodiment, the interaction of each rotamer with the entire remainder of the protein, i.e. both the entire template and all other rotamers, is done. However, as outlined above, it is possible to only model a portion of a protein, for example a domain of a larger protein, and thus in some cases, not all of the protein need be considered. The term "portion", or similar grammatical equivalents thereof, as used herein, with regard to a protein refers to a fragment of that protein. This fragment may range in size from 5-10 amino acid residues to the entire amino acid sequence minus one amino acid. Accordingly, the term "portion", as used



herein, with regard to a nucleic refers to a fragment of that nucleic acid. This fragment may range in size from 6-10 nucleotides to the entire nucleic acid sequence minus one nucleotide.

In a preferred embodiment, the first step of the computational processing is done by calculating two sets of interactions for each rotamer at every position: the interaction of the rotamer side chain with the template or backbone (the "singles" energy), and the interaction of the rotamer side chain with all other possible rotamers at every other position (the "doubles" energy), whether that position is varied or floated. It should be understood that the backbone in this case includes both the atoms of the protein structure backbone, as well as the atoms of any fixed residues, wherein the fixed residues are defined as a particular conformation of an amino acid.

Thus, "singles" (rotamer/template) energies are calculated for the interaction of every possible rotamer at every variable residue position with the backbone, using some or all of the scoring functions. Thus, for the hydrogen bonding scoring function, every hydrogen bonding atom of the rotamer and every hydrogen bonding atom of the backbone is evaluated, and the  $E_{HB}$  is calculated for each possible rotamer at every variable position. Similarly, for the van der Waals scoring function, every atom of the rotamer is compared to every atom of the template (generally excluding the backbone atoms of its own residue), and the  $E_{vdW}$  is calculated for each possible rotamer at every variable residue position. In addition, generally no van der Waals energy is calculated if the atoms are connected by three bonds or less. For the atomic solvation scoring function, the surface of the rotamer is measured against the surface of the template, and the  $E_{ss}$  for each possible rotamer at every variable residue position is calculated. The secondary structure propensity scoring function is also considered as a singles energy, and thus the total singles energy may contain an  $E_{ss}$  term. As will be appreciated by those in the art, many of these energy terms will be close to zero, depending on the physical distance between the rotamer and the template position; that is, the farther apart the two moieties, the lower the energy.

For the calculation of "doubles" energy (rotamer/rotamer), the interaction energy of each possible rotamer is compared with every possible rotamer at all other variable residue positions. Thus, "doubles" energies are calculated for the interaction of every possible rotamer at every variable residue position with every possible rotamer at every other variable residue position, using some or all of the scoring functions. Thus, for the hydrogen bonding scoring function, every hydrogen bonding atom of the first rotamer and every hydrogen bonding atom of every possible second rotamer is evaluated, and the  $E_{HB}$  is calculated for each possible rotamer pair for any two variable positions. Similarly, for the van der Waals scoring function, every atom of the first rotamer is compared to every atom of every possible second rotamer, and the  $E_{vdW}$  is calculated for each possible rotamer pair at every two variable residue positions. For the atomic solvation scoring function, the surface of the first rotamer is measured against the surface of every possible second rotamer, and the  $E_{ss}$  for each possible rotamer pair at every two variable residue positions is calculated. The secondary structure

propensity scoring function need not be run as a "doubles" energy, as it is considered as a component of the "singles" energy. As will be appreciated by those in the art, many of these double energy terms will be close to zero, depending on the physical distance between the first rotamer and the second rotamer; that is, the farther apart the two moieties, the lower the energy.

5 In addition, as will be appreciated by those in the art, a variety of force fields that can be used in the PDA calculations, including, but not limited to, Dreiding I and Dreiding II [Mayo et al, J. Phys. Chem. 94:8897 (1990)], AMBER [Weiner et al., J. Amer. Chem. Soc. 106:765 (1984) and Weiner et al., J. Comp. Chem. 106:230 (1986)], MM2 [Allinger, J. Chem. Soc. 99:8127 (1977), Liljefors et al., J. Com. Chem. 8:1051 (1987)]; MMP2 [Sprague et al., J. Comp. Chem. 8:581 (1987)]; CHARMM [Brooks et al., J. Comp. Chem. 106:187 (1983)]; GROMOS; and MM3 [Allinger et al., J. Amer. Chem. Soc. 111:8551 (1989)], OPLS-AA [Jorgensen et al., J. Am. Chem. Soc. 118:11225-11236 (1996); Jorgensen, W.L.; BOSS, Version 4.1; Yale University: New Haven, CT (1999)]; OPLS [Jorgensen et al., J. Am. Chem. Soc. 110:1657ff (1988); Jorgensen et al., J Am. Chem. Soc. 112:4768ff (1990)]; UNRES (United Residue Forcefield; Liwo et al., Protein Science 2:1697-1714 (1993); Liwo et al., Protein Science 2:1715-1731 (1993); Liwo et al., J. Comp. Chem. 18:849-873 (1997); Liwo et al., J. Comp. Chem. 18:874-884 (1997); Liwo et al., J. Comp. Chem. 19:259-276 (1998); Forcefield for Protein Structure Prediction (Liwo et al., Proc. Natl. Acad. Sci. U.S.A 96:5482-5485 (1999)]; ECEPP/3 [Liwo et al., J Protein Chem. 13(4):375-80 (1994)]; AMBER 1.1 force field (Weiner, et al., J. Am. Chem. Soc. 106:765-784); AMBER 3.0 force field (U.C. Singh et al., Proc. Natl. Acad. Sci. U.S.A.. 82:755-759); CHARMM and CHARMM22 (Brooks et al., J. Comp. Chem. 4:187-217); cvff3.0 [Dauber-Osguthorpe, et al., Proteins: Structure, Function and Genetics, 4:31-47 (1988)]; cff91 (Maple, et al., J. Comp. Chem. 15:162-182); also, the DISCOVER (cvff and cff91) and AMBER forcefields are used in the INSIGHT molecular modeling package (Biosym/MSI, San Diego California) and HARMM is used in the QUANTA molecular modeling package (Biosym/MSI, San Diego California), all of which  
25 are expressly incorporated by reference.

Once the singles and doubles energies are calculated and stored, the next step of the computational processing may occur. As outlined in U.S.S.N. 09/127,926 and PCT US98/07254, preferred embodiments utilize a Dead End Elimination (DEE) step, and preferably a Monte Carlo step.

30 PDA, viewed broadly, has three components that may be varied to alter the output (e.g. the primary library): the scoring functions used in the process; the filtering technique, and the sampling technique.

In a preferred embodiment, the scoring functions may be altered. In a preferred embodiment, the scoring functions outlined above may be biased or weighted in a variety of ways. For example, a bias towards or away from a reference sequence or family of sequences can be done; for example, a bias towards wild-type or homolog residues may be used. Similarly, the entire protein or a fragment of it

may be biased; for example, the active site may be biased towards wild-type residues, or domain residues towards a particular desired physical property can be done. Furthermore, a bias towards or against increased energy can be generated. Additional scoring function biases include, but are not limited to applying electrostatic potential gradients or hydrophobicity gradients, adding a substrate or binding partner to the calculation, or biasing towards a desired charge or hydrophobicity.

In addition, in an alternative embodiment, there are a variety of additional scoring functions that may be used. Additional scoring functions include, but are not limited to torsional potentials, or residue pair potentials, or residue entropy potentials. Such additional scoring functions can be used alone, or as functions for processing the library after it is scored initially. For example, a variety of functions derived from data on binding of peptides to MHC (Major Histocompatibility Complex) can be used to rescore a library in order to eliminate proteins containing sequences which can potentially bind to MHC, i.e. potentially immunogenic sequences.

In a preferred embodiment, a variety of filtering techniques can be done, including, but not limited to, DEE and its related counterparts. Additional filtering techniques include, but are not limited to branch-and-bound techniques for finding optimal sequences (Gordon and Mayo, Structure Fold. Des. 7:1089-98, 1999), and exhaustive enumeration of sequences.

As will be appreciated by those in the art, once an optimized sequence or set of sequences is generated, a variety of sequence space sampling methods can be done, either in addition to the preferred Monte Carlo methods, or instead of a Monte Carlo search. That is, once a sequence or set of sequences is generated, preferred methods utilize sampling techniques to allow the generation of additional, related sequences for testing.

These sampling methods can include the use of amino acid substitutions, insertions or deletions, or recombinations of one or more sequences. As outlined herein, a preferred embodiment utilizes a Monte Carlo search, which is a series of biased, systematic, or random jumps. However, there are other sampling techniques that can be used, including Boltzman sampling, genetic algorithm techniques and simulated annealing. In addition, for all the sampling techniques, the kinds of jumps allowed can be altered (e.g. random jumps to random residues, biased jumps (to or away from wild-type, for example), jumps to biased residues (to or away from similar residues, for example, etc.). Jumps where multiple residue positions are coupled (two residues always change together, or never change together), jumps where whole sets of residues change to other sequences (e.g., recombination). Similarly, for all the sampling techniques, the acceptance criteria of whether a sampling jump is accepted can be altered.

In addition, it should be noted that the preferred methods of the invention result in a rank ordered list of sequences; that is, the sequences are ranked on the basis of some objective criteria. However, as outlined herein, it is possible to create a set of non-ordered sequences, for example by generating a probability table directly (for example using SCMF analysis or sequence alignment techniques) that lists sequences without ranking them. The sampling techniques outlined herein can be used in either situation.

In a preferred embodiment, Boltzman sampling is done. As will be appreciated by those in the art, the temperature criteria for Boltzman sampling can be altered to allow broad searches at high temperature and narrow searches close to local optima at low temperatures (see e.g., Metropolis et al., J. Chem. Phys. 21:1087, 1953).

In a preferred embodiment, the sampling technique utilizes genetic algorithms, e.g., such as those described by Holland (Adaptation in Natural and Artificial Systems, 1975, Ann Arbor, U. Michigan Press). Genetic algorithm analysis generally takes generated sequences and recombines them computationally, similar to a nucleic acid recombination event, in a manner similar to "gene shuffling". Thus the "jumps" of genetic algorithm analysis generally are multiple position jumps. In addition, as outlined below, correlated multiple jumps may also be done. Such jumps can occur with different crossover positions and more than one recombination at a time, and can involve recombination of two or more sequences. Furthermore, deletions or insertions (random or biased) can be done. In addition, as outlined below, genetic algorithm analysis may also be used after the secondary library has been generated.

In a preferred embodiment, the sampling technique utilizes simulated annealing, e.g., such as described by Kirkpatrick et al. [Science, 220:671-680 (1983)]. Simulated annealing alters the cutoff for accepting good or bad jumps by altering the temperature. That is, the stringency of the cutoff is altered by altering the temperature. This allows broad searches at high temperature to new areas of sequence space, altering with narrow searches at low temperature to explore regions in detail.

In addition, as outlined below, these sampling methods can be used to further process a first set to generate additional sets of XA proteins.

The computational processing results in a set of optimized XA protein sequences. These optimized XA protein sequences are generally significantly different from the wild-type xylanase sequence from which the backbone was taken. That is, each optimized XA protein sequence preferably comprises at least about 3-10% variant amino acids from the starting or wild type sequence, with at least about 10-15% being preferred, with at least about 15-20% changes being more preferred and at least 25% being particularly preferred.

In a preferred embodiment, the XA proteins of the invention have 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 different residues from the *Bacillus circulans* xylanase sequence.

Thus, in the broadest sense, the present invention is directed to XA proteins that have xylanase activity. By "Xylanase activity" or "XA" herein is meant that the XA protein exhibits at least one, and preferably more, of the biological functions of a xylanase, as defined below. In one embodiment, the biological function of an XA protein is altered, preferably improved, over the corresponding biological activity of the *B. circulans* xylanase.

By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures, i.e., "analogs" such as peptoids [see Simon et al., Proc. Natl. Acad. Sci. U.S.A. 89(20:9367-71 (1992))], generally depending on the method of synthesis. Thus "amino acid", or "peptide residue", as used herein means both naturally occurring and synthetic amino acids. For example, homo-phenylalanine, citrulline, and noreleucine are considered amino acids for the purposes of the invention. "Amino acid" also includes imino acid residues such as proline and hydroxyproline. In addition, any amino acid representing a component of the XA proteins can be replaced by the same amino acid but of the opposite chirality. Thus, any amino acid naturally occurring in the L-configuration (which may also be referred to as the R or S, depending upon the structure of the chemical entity) may be replaced with an amino acid of the same chemical structural type, but of the opposite chirality, generally referred to as the D- amino acid but which can additionally be referred to as the R- or the S-, depending upon its composition and chemical configuration. Such derivatives have the property of greatly increased stability, and therefore are advantageous in the formulation of compounds which may have longer in vivo half lives, when administered by oral, intravenous, intramuscular, intraperitoneal, topical, rectal, intraocular, or other routes. In the preferred embodiment, the amino acids are in the (S) or L-configuration. If non-naturally occurring side chains are used, non-amino acid substituents may be used, for example to prevent or retard in vivo degradations. Proteins including non-naturally occurring amino acids may be synthesized or in some cases, made recombinantly; see van Hest et al., FEBS Lett 428:(1-2) 68-70 May 22 1998 and Tang et al., Abstr. Pap Am. Chem. S218:U138-U138 Part 2 August 22, 1999, both of which are expressly incorporated by reference herein.

Additionally, modified amino acids or chemical derivatives of amino acids of consensus or fragments of XA proteins, according to the present invention may be provided, which polypeptides contain additional chemical moieties or modified amino acids not normally a part of the protein. Covalent and non-covalent modifications of the protein are thus included within the scope of the present invention.

Such modifications may be introduced into an XA polypeptide by reacting targeted amino acid residues of the polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or terminal residues. The following examples of chemical derivatives are provided by way of illustration and not by way of limitation.

5 Aromatic amino acids may be replaced with D- or L-naphylalanine, D- or L-Phenylglycine, D- or L-2-thieneylalanine, D- or L-1-, 2-, 3- or 4-pyreneylalanine, D- or L-3-thieneylalanine, D- or L-(2-pyridinyl)-alanine, D- or L-(3-pyridinyl)-alanine, D- or L-(2-pyrazinyl)-alanine, D- or L-(4-isopropyl)-phenylglycine, D-(trifluoromethyl)-phenylglycine, D-(trifluoromethyl)-phenylalanine, D-p-fluorophenylalanine, D- or L-p-biphenylphenylalanine, D- or L-p-methoxybiphenylphenylalanine, D- or L-2-indole(alkyl)alanines, and  
 10 D- or L-alkylainines where alkyl may be substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, isopropyl, iso-butyl, sec-isotyl, iso-pentyl, non-acidic amino acids, of C1-C20.

Acidic amino acids can be substituted with non-carboxylate amino acids while maintaining a negative charge, and derivatives or analogs thereof, such as the non-limiting examples of (phosphono)alanine, (phosphono)glycine, (phosphono)leucine, (phosphono)isoleucine, (phosphono)threonine, or  
 15 (phosphono)serine; or sulfated (e.g.,  $-SO_3H$ ) threonine, serine, tyrosine.

Other substitutions may include unnatural hydroxylated amino acids that may be made by combining "alkyl" with any natural amino acid. The term "alkyl" as used herein refers to a branched or unbranched saturated hydrocarbon group of 1 to 24 carbon atoms, such as methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, t-butyl, octyl, decyl, tetradecyl, hexadecyl, eicosyl, tetracosyl and the like.  
 20 Preferred alkyl groups herein contain 1 to 12 carbon atoms. Also included within the definition of an alkyl group are cycloalkyl groups such as C5 and C6 rings, and heterocyclic rings with nitrogen, oxygen, sulfur or phosphorus. Alkyl also includes heteroalkyl, with heteroatoms of sulfur, oxygen, and nitrogen being preferred. Alkyl includes substituted alkyl groups. By "substituted alkyl group" herein is meant an alkyl group further comprising one or more substitution moieties. A preferred heteroalkyl  
 25 group is an alkyl amine. By "alkyl amine" or grammatical equivalents herein is meant an alkyl group as defined above, substituted with an amine group at any position. In addition, the alkyl amine may have other substitution groups, as outlined above for alkyl group. The amine may be primary ( $-NH_2R$ ), secondary ( $-NHR_2$ ), or tertiary ( $-NR_3$ ). Basic amino acids may be substituted with alkyl groups at any position of the naturally occurring amino acids lysine, arginine, ornithine, citrulline, or (guanidino)-acetic acid, or other (guanidino)alkyl-acetic acids, where "alkyl" is define as above. Nitrile derivatives  
 30 (e.g., containing the CN-moiety in place of COOH) may also be substituted for asparagine or glutamine, and methionine sulfoxide may be substituted for methionine. Methods of preparation of such peptid derivatives are well known to one skilled in the art.

In addition, any amide linkage in any of the XA polypeptides can be replaced by a ketomethylene moiety. Such derivatives are expected to have the property of increased stability to degradation by enzymes

Additional amino acid modifications of amino acids of XA polypeptides of the present invention may include the following: Cysteiny residues may be reacted with alpha-haloacetates (and corresponding amines), such as 2-chloroacetic acid or chloroacetamide, to give carboxymethyl or  
5 carboxyamidomethyl derivatives. Cysteiny residues may also be derivatized by reaction with compounds such as bromotrifluoroacetone, alpha-bromo-beta-(5-imidozoyl)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole.

10 Histidyl residues may be derivatized by reaction with compounds such as diethylprocarbonate e.g., at pH 5.5-7.0 because this agent is relatively specific for the histidyl side chain, and para-bromophenacyl bromide may also be used; e.g., where the reaction is preferably performed in 0.1M sodium cacodylate at pH 6.0.

15 Lysiny residues and amino terminal residues may be reacted with compounds such as succinic or other carboxylic acid anhydrides. Derivatization with these agents is expected to have the effect of reversing the charge of the lysiny residues. Other suitable reagents for derivatizing alpha-amino-containing residues include compounds such as imidoesters/e.g., as methyl picolinimate; pyridoxal phosphate; pyridoxal; chloroborohydride; trinitrobenzenesulfonic acid; O-methylisourea; 2,4 pentanedione; and transaminase-catalyzed reaction with glyoxylate.

20 Arginy residues may be modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin according to known method steps. Derivatization of arginine residues requires that the reaction be performed in alkaline conditions because of the high pKa of the guanidine functional group. Furthermore, these reagents may react with the groups of lysine as well as the arginine epsilon-amino group.

25 The specific modification of tyrosyl residues per se is well-known, such as for introducing spectral labels into tyrosyl residues by reaction with aromatic diazonium compounds or tetranitromethane. N-acetylimidizol and tetranitromethane may be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Carboxyl side groups (aspartyl or glutamyl) may be selectively modified by reaction with carbodiimides

(R'-N-C-N-R') such as 1-cyclohexyl-3-(2-morpholinyl- (4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethylpentyl) carbodiimide. Furthermore aspartyl and glutamyl residues may be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Glutamyl and asparaginyl residues may be frequently deamidated to the corresponding glutamyl and aspartyl residues. Alternatively, these residues may be deamidated under mildly acidic conditions. Either form of these residues falls within the scope of the present invention.

The xylanase may be from any number of organisms, with xylanase from *Bacillus circulans* being particularly preferred. Suitable organisms include, but are not limited to bacteria, archaeobacteria, yeast and fungi and plants. The GenBank accession numbers for a variety of endo-1,4-beta xylanases (xylanase) proteins and nucleic acids encoding same, include, but are not limited to: *Acidobacterium capsulatum* (JE0182); *Arabidopsis thaliana* (T00624); *Aspergillus awamori* (S48229); *Aspergillus kawachii* (BAA07264, BAA03576, BAA03575); *Aspergillus niger* (BAA07265, JT0608, JC1198); *Aspergillus tubigensis* (S49542); *Bacillus circulans* (S01734); *Bacillus polymyxa* (S19011); *Bacillus pumilus* (S00067, WWBSXP); *Bacillus sp.* (T17628, JC7103, I40356, JD0003); *Bacillus subtilis* (H69735, G69849, F69829; I40569); *Bacillus stearothermophilus* (I40570, I39760); *Bacteroides ovatus* (S55892); barley (T06195, T06191); *Caldicellulosiruptor sp.* (T31085, T31082); *Caldocellum saccharolyticum* (T30909, A60154); *Cellulomonas fimi* (T31351, I40712); *Cellvibrio mixtus* (S59631); *Chaetomium gracile* (BAA08650, BAA08649, S71472, S71473); *Clostridium acetobutylium* (S12745); *Clostridium stercorarium* (JC2484, JQ1935); *Clostridium thermocellum* (S54975, A31842); *Cryptococcus sp.* (JC4909); *Dictyoglomus thermophilum* (T08469); *Emericella nidulans* (JC5034, S57477, S57469); *Filobasidium floriforme* (JS0734); *Humicola grisea* (JC5861); *Humicola insolens* (S43919); *Neocallimastix frontalis* (S48865); *Neocallimastix patriciarum* (S43846; S24754); *Nocardiosis dassonvillei* (PQ0203); *Penicillium chrysogenum* (JN0575); *Pseudomonas fluorescens* (S59633, S59634, S13391, S06047); *Ruminococcus flavefaciens* (S51592, S20907); *Ruminococcus sp.* (S58235); *Schizophyllum commune* (A44597); *Streptomyces sp.* (S70615, S47512); *Streptomyces coelicolor* (T37005, CAB61738); *Streptomyces lividans* (JS0591, JS0590, JS0589); *Streptomyces roseiscleroticus* (C57001, A57001, B57001, D57001); *Streptomyces thermoviolaceus* (B43937, A43937); *Thermoanaerobacterium saccharolyticum* (A48490); thermophilic bacterium RT8.B4 (S41788); *Thermoascus Aurantiacus* (1TAXA, 1TIXA, AAF24127, CAB65468); *Thermotoga maritima* (strain MSB8) (B72423, S61311); *Thielavia terrestris* (A44598); *Trichoderma viride* (A44595, A44594); *Trichoderma harzianum* (A44593); *Trichoderma reesei* (S39883, S39155, S39154).

The XA proteins of the invention exhibit at least one biological function of a xylanase. By "xylanase" herein is meant a wild type xylanase or an allelic variant thereof. Included within this definition are, for example, the *B. circulans* xylanase and the *B. subtilis* xylanase; these two enzymes are identical



except at position 147, where *B. circulans* has a threonine and *B. subtilis* has a serine. In its broadest term, xylanase refers to all forms of xylanase that are active in accepted xylanase assays.

In a preferred embodiment, a xylanase belongs to the Family 11 of xylanases. An enzyme is classified in family 11 if it possesses the conserved amino acid residues common to Family 11, including the two glutamic acid residues serving as the essential catalytic residues [see Wakarchuk et al., Protein Sci. 3:467-475 (1994)].

The XA proteins of the invention exhibit at least one biological function of a xylanase. By "biological function" or "biological property" herein is meant any one of the properties or functions of a xylanase including, but not limited to, the ability to hydrolyze pulp xylan, the ability to hydrolyze pure xylan, and the ability to hydrolyze cellulose, and the ability to be secreted.

"Hydrolyzing xylan" herein means hydrolyzing the 1,4-beta-D-xyloside bond of xylan to thereby produce reducing sugars of xylooligosaccharides.

"Xylan" as used herein, includes birch xylan, oat spelt xylan, as well as xylan-containing materials, including hardwood kraft pulp and oat spelt pulp.

All of these XA proteins will exhibit at least 50% of the biological activity as the wild type *B. circulans* xylanase. More preferred are XA proteins that exhibit at least 75%, even more preferred are XA proteins that exhibit at least 90%, and most preferred are XA proteins that exhibit more than 100% of the biological activity as the wild type xylanase. Xylanase assays are described in U.S. Patent Nos. 5,405,769; 5,736,384; 5,759,840; Arase et al. [FEB Lett. 316(2):123-7 (1993)]; Wakarchuk et al. [Protein Sci. 3(3):467-75 (1994); Protein Eng. 7(11):1379-86 (1994)]; and references cited therein, all of which are expressly incorporated by reference.

In one embodiment, at least one biological property of the XA protein is altered when compared to the same property of *B. circulans* xylanase. As outlined above, the invention provides XA nucleic acids encoding XA polypeptides. The XA polypeptide preferably has at least one property, which is substantially different from the same property of the corresponding naturally occurring *B. circulans* xylanase polypeptide. The property of the XA polypeptide is the result the PDA analysis of the present invention.

The term "altered property" or grammatical equivalents thereof in the context of a polypeptide, as used herein, refer to any characteristic or attribute of a polypeptide that can be selected or detected and compared to the corresponding property of a naturally occurring protein. These properties include, but

are not limited to oxidative stability, substrate specificity, substrate binding or catalytic activity, thermostability, thermophilicity, alkaline stability, alkalophilicity, pH activity profile, resistance to proteolytic degradation,  $K_m$ ,  $k_{cat}$ ,  $K_m/k_{cat}$  ratio, kinetic association ( $K_{on}$ ) and dissociation ( $K_{off}$ ) rate, protein folding, ability to be secreted, ability to be modified by phosphorylation or glycosylation

5 Unless otherwise specified, a substantial change in any of the above-listed properties, when comparing the property of an XA polypeptide to the property of a naturally occurring *B. circulans* xylanase, is at least a 10%, preferably at least a 20%, more preferably, 50%, more preferably at least a 2-fold increase or decrease.

10 A change in oxidative stability is evidenced by at least about 20%, more preferably at least 50% increase of activity of an XA protein when exposed to various oxidizing conditions as compared to that of *B. circulans* xylanase. Oxidative stability is measured by known procedures.

15 The term "thermophilicity", as used herein is defined as the ability of an enzyme to be active at a high temperature. For example, a XA protein has more thermophilicity than *B. circulans* xylanase if it is more efficient in hydrolyzing a substrate, such as xylan, at a temperature higher than the optimum for the *B. circulans* xylanase. Thermophilicity relates to enzyme activity in the presence of substrate. In the present invention, the substrate can be pulp xylan or purified xylan. The XA protein will have a temperature optimum which is at least 1°C greater than the temperature optimum for the *B. circulans* xylanase, preferably at least 3°C- 5°C, more preferably at least 6°C, 7°C, 8°C, 9°C, 10°C, 11°C, 12°C, 13°C, 14°C, 15°C, 16°C, 17°C, 18°C, 19°C, 20°C, 21°C, 22°C, 23°C, 24°C, or 25°C. In some  
20 embodiments, the shift in temperature optimum may be as high 30°C to 35°C and even up to 60°C. Included within this definition of thermophilicity is a XA protein, which has at least one temperature at which it hydrolyzes a substrate more efficient than the *B. circulans* xylanase. In another example, a shift in temperature of 5°C means that the XA enzyme has the same activity as the wild type *B. circulans* xylanase, however at a 5°C higher temperature. Generally, thermophilicity is measured by  
25 known procedures.

30 The term "substrate", as used herein refers to a substrate of an enzyme. In a preferred embodiment, the substrate is xylan and the enzyme is xylanase or a XA protein. In one aspect of this embodiment the substrate is a derivative of xylan. Most xylanase enzymes are effective at higher temperatures in the hydrolysis of pure xylan than in the treatment of pulp. This is due to a combination of factors relating to the substrates (i.e. inhibitors present in the pulp) and to the length of time, pH, and other aspects of the procedures used to carry out the tests. Quantitative measures of thermophilicity refer herein to xylan substrates unless otherwise indicated.

The term "thermostability" or "thermal stability" or grammatical equivalents thereof, as used herein, is defined as the ability of an enzyme to be stored or incubated at a high temperature in the absence of substrate, such as xylan, and then exhibit xylanase activity when returned to standard assay conditions. For example, an XA protein is more thermostable than *B. circulans* xylanase if it can be held at 65°C- 70°C for a period of time and still retains a greater percentage of its activity when compared to the *B. circulans* xylanase, which loses all or most of its activity after 24 hours at 65°C- 70°C. Thus, in contrast to thermophilicity, thermostability relates to the enzyme activity remaining after incubation in the absence of a substrate, such as xylan [Mathrani and Ahring, Appl. Microbiol. Biotechnol. 38:23-27 (1992)].

A change in thermostability is evidenced by at least about a 5% or greater increase or decrease (preferably increase) in the half life of the activity of an XA protein when exposed to a relatively high temperature and neutral pH as compared to that of *B. circulans* xylanase. Generally, thermal stability is measured by known procedures.

The term "alkalophilicity", as used herein is defined as the ability of an enzyme to be active at a high (alkaline) pH. For example, an XA protein has more alkalophilicity than *B. circulans* xylanase if it is more efficient in hydrolyzing a substrate, such as xylan, at a pH higher than the optimum for the *B. circulans* xylanase. Alkalophilicity is analogous to thermophilicity and relates to enzyme activity in the presence of a substrate, such as xylan. The XA protein will have a pH optimum which is at least greater by 0.1, preferably at least greater than 0.3-0.5, more preferably at least greater than 0.5-1.0, 1-0-2.0, 2.0-3.0, 3.0-4.0, 4.0-5.0, or 5.0-6.0. Included within this definition of alkalophilicity is a XA protein, which has at least one pH at which it hydrolyzes a substrate more efficient than the *B. circulans* xylanase. In another example, a shift in pH of 3.0 means that the XA enzyme has the same activity as the wild type *B. circulans* xylanase, however at a pH that is 3.0 higher. Generally, alkalophilicity is measured by known procedures.

Accordingly, the term "alkaline stability" or grammatical equivalents thereof, as used herein refers ability of an enzyme to be stored or incubated at a high pH in the absence of substrate, such as xylan, and then exhibit xylanase activity when returned to standard assay conditions. For example, an XA protein is more alkaline stable than *B. circulans* xylanase if it can be held at pH 8 and retain all or most of its activity, while *B. circulans* xylanase loses all or most of its activity after being held at pH 8 for the same time. Thus, in contrast to alkalophilicity, alkaline stability relates to the enzyme activity remaining after incubation in the absence of a substrate, such as xylan.

A change in alkaline stability is evidenced by at least about a 5% or greater increase or decrease (preferably increase) in the activity of an XA protein when exposed to increasing or decreasing pH conditions as compared to that of *B. circulans* xylanase. Generally, alkaline stability is measured by known procedures.

5 Production, purification procedures and assays for xylanases, including (i) standard assays for the measurement of enzymatic activity, (ii) assays for determining the temperature/activity profile of xylanases, (iii) assays for comparing the thermophilicity of xylanases (iv) assays for determining the pH/activity profile of xylanases, (v) assays for measuring the thermostability of xylanases, (vi) assays for evaluating the performance of xylanases in treatment of pulp, (vii) bleaching of pulp are described  
10 in US patents Nos 5,759,840, 5,736,384 and 5,405,769 and in the references cited therein, all of which are expressly incorporated by reference. A preferred xylanase assay that can be used for high throughput assays is described by Green et al. [Analytical Biochemistry 182:197-199 (1989); hereby expressly incorporated by reference].

15 Similarly, XA proteins, for example are experimentally tested and validated in *in vitro* assays. Suitable assays include, but are not limited to e.g., examining their binding affinity to naturally occurring substrates or variant substrates. Quantitative comparison are made comparing kinetic and equilibrium binding constants for the *B. circulans* xylanase to a substrate and of the XA proteins to a substrate. The kinetic association rate ( $K_{on}$ ) and dissociation rate ( $K_{off}$ ), and the equilibrium binding constants ( $K_d$ )  
20 can be determined using surface plasmon resonance on a BIAcore instrument following the standard procedure in the literature [Pearce et al., Biochemistry 38:81-89 (1999)]. Comparing the binding constant between *B. circulans* xylanase and a substrate to the binding constant of an XA protein and the same substrate, the sensitivity and specificity of the XA protein can be determined. Preferably, binding affinity of the XA protein to a substrate increases relative to the *B. circulans* xylanase.

25 A change in substrate specificity is defined as a difference between the  $k_{cat}/K_m$  ratio of the naturally occurring protein, such as *B. circulans* xylanase and that of a variant thereof, such as an XA protein of the invention. The  $k_{cat}/K_m$  ratio is generally a measure of catalytic efficiency. Generally, the objective will be to generate variants of naturally occurring proteins with greater (numerically large)  $k_{cat}/K_m$  ratio for a given substrate when compared to that of the naturally occurring protein, thereby enabling the use of the protein to more efficiently act on a target substrate. An increase in  $k_{cat}/K_m$   
30 ratio for one substrate may be accompanied by a reduction in  $k_{cat}/K_m$  ratio for another substrate. This is a shift in substrate specificity and variants of naturally occurring proteins exhibiting such shifts have utility where the naturally occurring protein is undesirable, e.g., to prevent undesired hydrolysis of

a particular substrate in an admixture of substrates.  $K_m$  and  $k_{cat}$  are measured in accord with known procedures.

The XA proteins and nucleic acids of the invention are distinguishable from naturally occurring *B. circulans* xylanase. By "naturally occurring" or "wild type" or grammatical equivalents, herein is meant an amino acid sequence or a nucleotide sequence that is found in nature and includes allelic variations; that is, an amino acid sequence or a nucleotide sequence that usually has not been intentionally modified. Accordingly, by "non-naturally occurring" or "synthetic" or "recombinant" or grammatical equivalents thereof, herein is meant an amino acid sequence or a nucleotide sequence that is not found in nature; that is, an amino acid sequence or a nucleotide sequence that usually has been intentionally modified. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell or organism, it will replicate non-recombinantly, i.e., using the in vivo cellular machinery of the host cell rather than in vitro manipulations, however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purpose of the invention. Representative amino acid and nucleotide sequences of a naturally occurring *B. circulans* xylanase are shown in Figure 1. It should be noted that unless otherwise stated, all positional numbering of XA proteins and XA nucleic acids is based on these sequences. That is, as will be appreciated by those in the art, an alignment of *B. circulans* xylanase and XA proteins can be done using standard programs, as is outlined below, with the identification of "equivalent" positions between the two proteins. Thus, the XA proteins and nucleic acids of the invention are non-naturally occurring; that is, they do not exist in nature.

Thus, in a preferred embodiment, the XA protein has an amino acid sequence that differs from a wild-type *B. circulans* xylanase sequence by at least 3% of the residues. That is, the XA proteins of the invention are less than about 97% identical to a *B. circulans* xylanase amino acid sequence.

Accordingly, a protein is an "XA protein" if the overall homology of the protein sequence to the amino acid sequence shown in Figure 1A or Figure 1B is preferably less than about 97%, more preferably less than about 95%, even more preferably less than about 90% and most preferably less than 85%. In some embodiments the homology will be as low as about 75 to 80%. Stated differently, based on the sequence of the secreted form of *B. circulans* xylanase of 186 residues (see Figure 1A and Figure 1B), XA proteins have at least about 5 to 6 residues that differ from the *B. circulans* xylanase sequence (3%), with XA proteins having from 5 residues to upwards of 33 residues or even upwards to 79 residues being different from the *B. circulans* xylanase sequence. Preferred XA proteins have 5-30 different residues with from about 5 to about 15 being particularly preferred (that is, about 3-8% of the protein is not identical to *B. circulans* xylanase).

In another preferred embodiment, XA proteins have 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, or 40 different residues from the *B. circulans* xylanase sequence.

Homology in this context means sequence similarity or identity, with identity being preferred. As is known in the art, a number of different programs can be used to identify whether a protein (or nucleic acid as discussed below) has sequence identity or similarity to a known sequence. Sequence identity and/or similarity is determined using standard techniques known in the art, including, but not limited to, the local sequence identity algorithm of Smith & Waterman, Adv. Appl. Math., 2:482 (1981), by the sequence identity alignment algorithm of Needleman & Wunsch, J. Mol. Biol., 48:443 (1970), by the search for similarity method of Pearson & Lipman, Proc. Natl. Acad. Sci. U.S.A., 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, WI), the Best Fit sequence program described by Devereux et al., Nucl. Acid Res., 12:387-395 (1984), preferably using the default settings, or by inspection. Preferably, percent identity is calculated by FastDB based upon the following parameters: mismatch penalty of 1; gap penalty of 1; gap size penalty of 0.33; and joining penalty of 30, "Current Methods in Sequence Comparison and Analysis," Macromolecule Sequencing and Synthesis, Selected Methods and Applications, pp 127-149 (1988), Alan R. Liss, Inc.

An example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle, J. Mol. Evol. 35:351-360 (1987); the method is similar to that described by Higgins & Sharp CABIOS 5:151-153 (1989). Useful PILEUP parameters including a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps.

Another example of a useful algorithm is the BLAST algorithm, described in: Altschul et al., J. Mol. Biol. 215, 403-410, (1990); Altschul et al., Nucleic Acids Res. 25:3389-3402 (1997); and Karlin et al., Proc. Natl. Acad. Sci. U.S.A. 90:5873-5787 (1993). A particularly useful BLAST program is the WU-BLAST-2 program which was obtained from Altschul et al., Methods in Enzymology, 266:460-480 (1996); [http://blast.wustl.edu/blast/ README.html](http://blast.wustl.edu/blast/README.html)]. WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span =1, overlap fraction = 0.125, word threshold (T) = 11. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the

composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity.

An additional useful algorithm is gapped BLAST as reported by Altschul et al., Nucl. Acids Res., 25:3389-3402. Gapped BLAST uses BLOSUM-62 substitution scores; threshold  $T$  parameter set to 9; the two-hit method to trigger ungapped extensions; charges gap lengths of  $k$  a cost of  $10+k$ ;  $X_u$  set to 16, and  $X_g$  set to 40 for database search stage and to 67 for the output stage of the algorithms. Gapped alignments are triggered by a score corresponding to  $\sim 22$  bits.

A % amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "longer" sequence in the aligned region. The "longer" sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-Blast-2 to maximize the alignment score are ignored).

In a similar manner, "percent (%) nucleic acid sequence identity" with respect to the coding sequence of the polypeptides identified herein is defined as the percentage of nucleotide residues in a candidate sequence that are identical with the nucleotide residues in the coding sequence of the cell cycle protein. A preferred method utilizes the BLASTN module of WU-BLAST-2 set to the default parameters, with overlap span and overlap fraction set to 1 and 0.125, respectively.

The alignment may include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer amino acids than the protein encoded by the sequence of Figure 1, it is understood that in one embodiment, the percentage of sequence identity will be determined based on the number of identical amino acids in relation to the total number of amino acids. Thus, for example, sequence identity of sequences shorter than that shown in Figure 1, as discussed below, will be determined using the number of amino acids in the shorter sequence, in one embodiment. In percent identity calculations relative weight is not assigned to various manifestations of sequence variation, such as, insertions, deletions, substitutions, etc.

In one embodiment, only identities are scored positively (+1) and all forms of sequence variation including gaps are assigned a value of "0", which obviates the need for a weighted scale or parameters as described below for sequence similarity calculations. Percent sequence identity can be calculated, for example, by dividing the number of matching identical residues by the total number of residues of the "shorter" sequence in the aligned region and multiplying by 100. The "longer" sequence is the one having the most actual residues in the aligned region.

Thus, XA proteins of the present invention may be shorter or longer than the amino acid sequence shown in Figure 1A. Thus, in a preferred embodiment, included within the definition of XA proteins are portions or fragments of the sequences depicted herein. Fragments of XA proteins are considered XA proteins if a) they share at least one antigenic epitope; b) have at least the indicated homology; c) and preferably have XA biological activity as defined herein.

In a preferred embodiment, as is more fully outlined below, the XA proteins include further amino acid variations, as compared to the wild type *B. circulans* xylanase, than those outlined herein. In addition, as outlined herein, any of the variations depicted herein may be combined in any way to form additional novel XA proteins.

In addition, XA proteins can be made that are longer than those depicted in the figures, for example, by the addition of epitope or purification tags, as outlined herein, or the addition of other fusion sequences.

It is well known in the art that xylanase also has uses in non-pulp applications. For example, xylanases have been reported to be useful in clarifying juice and wine [Zeikus et al., ACS Symp. Ser. 460:36-51 (1991); Beily, ACS Symp. Ser. 460:408-416 (1991); Woodward, Top Enzyme Ferment. Biotechnol. 8:9-30 (1984)]; extracting coffee, plant oils and starch [Beily, supra; Woodward supra; McCleary, Int. J. Biol. Macromol. 8:349-354 (1986)]; for the production of food thickeners (Zeikus et al., supra); altering texture in bakery products, e.g., improving the quality of dough, help bread rise and processing of wheat and corn for starch production (Maat et al. *In Xylans and xylanases*, eds. Visseret al., Elsevier Sci pub., Amsterdam. ISBN 0-444-894-772 (1992); McCleary, supra; Krishnarau et al., J. Food Sci. 59:1251-1254 (1994); US Patent No. 5,306,633); for use as animal food additives to aid in the digestibility of feedstuffs; and in the washing of super precision devices and semiconductors [Takayuki et al., U.S. Pat. No. 5,078,802). Several of these application could benefit from a thermostable xylanase, for example, food processing at elevated temperatures.

Due to the sequence homology among related xylanases, one could expect that the introduction of mutations into one member of the xylanase family, for example, as demonstrated in the examples below for *B. circulans* xylanase, could be extended to other xylanases from bacteria, archaeobacteria, yeast, fungi or plants and produce similar effects.

In a preferred embodiment, the xylanase used for PDA design is *Bacillus subtilis* xylanase (Figure 16A); *Bacillus pumilus* xylanase (Figure 16B); *Streptomyces lividans* xylanase B (Figure 16C); *Streptomyces lividans* xylanase C (Figure 16D); *Clostridium acetobutylicum* xylanase (Figure 16E);



*Schizophyllum commune* xylanase (Figure 16F); *Trichoderma viride* xylanase (Figure 16G);  
*Trichoderma harzianum* xylanase (Figure 16H); *Trichoderma reesei* xyn I xylanase (Figure 16 I);  
*Trichoderma reesei* xyn II xylanase (Figure 16J); *Paecilomyces variotii* xylanase (Figure 16K);  
*Thermomyces lanuginosus* xylanase (Figure 16L); *Aspergillus niger* xylanase (Figure 16M);  
5 *Aspergillus awamori* var. *kawachi* xylanase A (Figure 16N); *Aspergillus awamori* var. *kawachi*  
xylanase (Figure 16O); *Neocalimastix patriciarum* xylanase (Figure 16P);  
*Cochliobolus carbonum* xylanase (Figure 16Q); *Clostridium stercorarium* xylanase (Figure 16R);  
*Ruminococcus flavefaciens* xylanase (Figure 16S); *Fibrobacter succinogenes* xylanase (Figure 16T);  
*Aspergillus tubigensis* xylanase (Figure 16U); or *Bacillus* sp. strain 41M-1 xylanase (Figure 16V).

10 In a preferred embodiment, the PDA design is performed on the *Trichoderma reesei* xylanase (xynII)  
sequence. The structure of the fungal xylanase from *Trichoderma reesei* (xynII) as solved by Torronen  
and Rouvinen [Biochemistry 34:847 (1995)] was taken from the PDB server, entry 1XYP.

In another preferred embodiment, the xylanase used for PDA design is *Bacillus* sp. B2113.

15 In a particularly preferred embodiment, the xylanase used for PDA design is *B. circulans* xylanase  
(PDB entry 1XNB).

The XA proteins of the invention comprise variable amino acid residues in core residues, in regions  
around D83, in the helix region and around the active site region.

In a preferred embodiment, the XA proteins comprise variable amino acid residues in core residues.

20 *B. circulans* xylanase core residues are as follows: positions 26, 28, 30, 36, 38, 51, 53, 55, 58, 62, 64,  
66, 68, 70, 72, 77, 79, 81, 105, 107, 130, 142, 144, 146, 153, 169, 171, 173, 176, 178, 180, 182, and  
184 (see Figure 3). Accordingly, in a preferred embodiment, XA proteins have variable positions  
selected from these positions.

25 In a preferred embodiment, XA proteins have variable positions selected solely from core residues of  
*B. circulans* xylanase. Alternatively, at least a majority (51%) of the variable positions are selected  
from core residues, with at least about 75% of the variable positions being preferably selected from  
core residue positions, and at least about 90% of the variable positions being particularly preferred. A  
specifically preferred embodiment has only core variable positions altered as compared to *B. circulans*  
xylanase.

Particularly preferred embodiments where XA proteins have variable positions as compared to *B. circulans xylanase* comprise the CORE region, as shown in Figure 4.

In one embodiment, the variable core positions are altered to any of the other 19 amino acids. In a preferred embodiment, the variable core residues are chosen from Ala, Val, Phe, Ile, Leu, Tyr, Trp and Met. In another preferred embodiment, the variable core residues are chosen from Ala, Val, Leu, Ile, Phe, Tyr, and Trp plus the original wild type residue.

In a preferred embodiment, the XA protein of the invention has a sequence that differs from a wild-type *B. circulans xylanase* protein in at least one amino acid position selected from positions 5, 7, 11, 26, 28, 30, 36, 37, 38, 39, 51, 53, 55, 58, 62, 63, 64, 65, 66, 67, 68, 70, 71, 72, 77, 79, 80, 81, 82, 83, 84, 85, 88, 95, 96, 98, 100, 101, 102, 103, 105, 107, 109, 110, 115, 118, 125, 128, 129, 130, 132, 136, 138, 142, 144, 146, 147, 148, 149, 150, 152, 153, 156, 157, 158, 160, 161, 164, 167, 168, 169, 170, 171, 173, 176, 178, 180, 182, and 184; see also Figure 3, which outlines sets of amino acid positions.

In another preferred embodiment, the XA protein of the invention has a sequence that differs from a wild type *B. circulans xylanase* sequence in at least one amino acid position selected from the positions 26, 28, 30, 36, 38, 51, 53, 55, 58, 62, 64, 66, 68, 70, 72, 77, 79, 81, 105, 107, 130, 142, 144, 146, 153, 169, 171, 173, 176, 178, 180, 182, and 184 (see Figure 3).

In one aspect of this embodiment, preferred amino acid changes within the CORE region are as follows (see Figures 4A): Y26F; V28I; V28A; V28S; V28L; V28W; W30F; W30Y; F36L; F36Y; V38I; I51L; I51V; Y53F; Y53W; A55S; W58F; W58A; W58S; G64V; G64A; L68V; I77V; I77L; Y79F; Y79W; V81I; Y105F; I107V; I107L; S130A; A142L; A142S; A142V; I144V; I144L; F146I; F146Y; F146V; W153L; M169L; T171L; T171I; T171V; G173A; S176A; S180A; S180F; V182I; and V182L. These may be done either individually or in combination, with any combination possible. However, as outlined herein, preferred embodiments utilize at least five, and preferably more variable positions in each XA protein.

In one particularly preferred embodiment, a preferred XA protein comprises the following changes (see Figure 4B); Y26F, V28I, W30F, Y53F, W58F, G64V, Y79F, Y105F, A142L, T171L, S176A, S180A, and V182I.

Particularly preferred embodiments where XA proteins have variable positions as compared to *B. circulans xylanase* comprise the 'Region around D83', as shown in Figures 5-10.

In a preferred embodiment, XA proteins have variable positions selected solely from 'Regions around D83' residues of *B. circulans xylanase*. Alternatively, at least a majority (51%) of the variable positions are selected from 'Regions around D83' residues, with at least about 75% of the variable positions being preferably selected from 'Regions around D83' residue positions, and at least about 90% of the variable positions being particularly preferred. A specifically preferred embodiment has only 'Regions around D83' variable positions altered as compared to *B. circulans xylanase*.

In one embodiment, the variable 'Region around D83' positions are altered to any of the other 19 amino acids. In a preferred embodiment, the variable core residues are chosen from Ala, Val, Phe, Ile, Leu, Tyr, Trp and Met. In another preferred embodiment, the variable core residues are chosen from Ala, Val, Leu, Ile, Phe, Tyr, and Trp plus the original wild type residue. In another embodiment, the variable residues were chosen from boundary rotamers (Ala, Val, Leu, Ile, Phe, Trp, Asp, Asn, Glu, Gln, Lys, Ser, Thr, His, Arg, Met).

In another preferred embodiment, the XA protein of the invention has a sequence that differs from a wild type *B. circulans xylanase* sequence in at least one amino acid position selected from the positions 53, 66, 67, 68, 81, 82, 83, 84, 85, 101, 105, 132, 136, 138, 142, 144, 149, and 169 (see Figure 3).

In one aspect of this embodiment, preferred amino acid changes within the 'Region around D83' are as follows (see Figures 5A, 6A, 9A, 10A): Y53F; Y53W; L66F; T67A; T67D; T67S; T67N; L68V; L68I; V81A; V82L; V82T; V82D; D83V; D83F; D83T; S84V; S84A; S84T; S84D; W85F; W85Y; D101N; D101A; D101S; Y105F; R132M; R132L; R132A; R132S; R136M; R136K; R136L; R136I; R136F; R136E; R136A; T138V; T138D; T138L; T138A; T138S; T138N; T138Hsp; A142L; A142I; A142V; A142F; I144L; I144A; H149F; H149Y; and M169L. These may be done either individually or in combination, with any combination possible. However, as outlined herein, preferred embodiments utilize at least five, and preferably more variable positions in each XA protein.

In one particularly preferred aspect of this embodiment, a preferred XA protein comprises the following changes (see Figures 5B and 6B): Y53F, D83V, S84V, W85F, Y105F, R132M, R136M, A142L, I144L, and H149F.

In another preferred aspect of this embodiment, a preferred XA protein comprises the following changes (see Figure 9B): Y53F, D83V, S84V, W85F, Y105F, R132A, R136L, A142L, I144L, and H149F.

In another preferred aspect of this embodiment, a preferred XA protein comprises the following changes (see Figure 10B): Y53F, D83V, S84A, W85F, Y105F, R132A, R136L, A142L, I144L, and H149F.

5 In another preferred embodiment, the XA protein of the invention has a sequence that differs from a wild type *B. circulans* xylanase sequence in at least one amino acid position selected from the positions 53, 66, 68, 81, 83, 84, 101, 105, 132, 136, 138, 142, 144, 149, and 169 (see Figure 3).

In one aspect of this embodiment, preferred amino acid changes within the 'Region around D83' are as follows (see Figures 7A, 8A): Y53F; Y53W; L66F; L68V; L68I; V81A; D83V; D83T, D83A, D83E; D83F; S84V; S84A; S84T; S84D; D101N; D101A; D101S; Y105F; R132M; R132L; R132A; R132S; 10 R132D; R132E; R136M; R136K; R136L; R136E; R136F; R136E; T138V; T138D; T138L; T138A; T138S; T138N; T138Hsp; A142L; A142I; A142V; I144L; I144V; I144A; H149F; H149Y; H149Hsp; and M169L. These may be done either individually or in combination, with any combination possible. However, as outlined herein, preferred embodiments utilize at least five, and preferably more variable positions in each XA protein.

15 In one particularly preferred aspect of this embodiment, a preferred XA protein comprises the following changes (see Figures 7B and 8B): Y53F, D83V, S84V, Y105F, R132M, R136M, A142L, I144L, and H149F.

Particularly preferred embodiments where XA proteins have variable positions as compared to *B. circulans* xylanase comprise the 'Helix region', as shown in Figures 11-13.

20 In a preferred embodiment, XA proteins have variable positions selected solely from 'Helix Region' residues of *B. circulans* xylanase. Alternatively, at least a majority (51%) of the variable positions are selected from 'Helix Region' residues, with at least about 75% of the variable positions being preferably selected from 'Helix Region' residue positions, and at least about 90% of the variable positions being particularly preferred. A specifically preferred embodiment has only "Helix Region" 25 variable positions altered as compared to *B. circulans* xylanase.

In one embodiment, the variable 'Helix Region' positions are altered to any of the other 19 amino acids. In a preferred embodiment, the variable core residues are chosen from Ala, Val, Phe, Ile, Leu, Tyr, Trp and Met. In another preferred embodiment, the variable core residues are chosen from Ala, Val, Leu, Ile, Phe, Tyr, and Trp plus the original wild type residue. In another embodiment, the variable

residues were chosen from boundary rotamers (Ala, Val, Leu, Ile, Phe, Trp, Asp, Asn, Glu, Gln, Lys, Ser, Thr, His, Arg, Met).

In another preferred embodiment, the XA protein of the invention has a sequence that differs from a wild type *B. circulans* xylanase sequence in at least one amino acid position selected from the positions: 70, 72, 77, 79, 81, 95, 96, 98, 100, 101, 102, 103, 105, 107, 109, 128, 130, 132, 144, 146, 147, 148, 149, 150, 152, 153, 156, 157, 158, 160, 161, 164, and 167 (see Figure 3).

In one aspect of this embodiment, preferred amino acid changes within the 'Helix Region' are as follows (see Figures 11A, 12A): I77L; I77V; Y79F; V81I; K95L; K95I; K95E; K95Q; K95V; K95R; G96S; V98T, V98D; V98A; V98E; V98T; V98L; S100V; S100A; G102D; G103I; Y105F; I107L; T109V; T109I; T109K; T109E; T109Q; T109L; T109D; T109R; Y1128V; Y128I; Y128L; S130A; R132A; I144L; I144V; T147I; T147D; T147E; T147V; T147Q; T147L; T147A; T147K; T147N; T147S N148E; N148Q; N148K; N148R; N148D; N148A; N148L; N148S; H149F; V150I; A152S; A152Y; A152W; A152D; W153F; H156Y, H156F; H156E; H156V; H156I; H156L; H156Q; H156N; H156K; M158I; M158L; M158L; M158V; M158E; L160F; and Q167E. These may be done either individually or in combination, with any combination possible. However, as outlined herein, preferred embodiments utilize at least five, and preferably more variable positions in each XA protein.

In one particularly preferred aspect of this embodiment, a preferred XA protein comprises the following changes (see Figures 11B); Y79F, G96S, V98T, S100V, G102D, G103I, Y105F, T109I, Y128V, S130A, R132A, I144L, T147I, N148E, H149F, V150I, A152S, H156Y, M158I, L160F, and Q167E.

In another particularly preferred aspect of this embodiment, a preferred XA protein comprises the following changes (see Figures 12B); Y79F, S100V, T109I, Y128V, T147I, N148E, V150I, A152S, H156Y, M158I, L160F, and Q167E.

In another particularly preferred aspect of this embodiment, a preferred XA protein comprises the following changes (see Figures 13B); Y79F, G96S, V98T, S100V, Y105F, T109I, Y128V, S130A; R132M; I144I; T147I, N148E, H149F; A152S, H156Y, M158I, and Q167E.

Particularly preferred embodiments where XA proteins have variable positions as compared to *B. circulans* xylanase comprise the 'Active Site Region', as shown in Figures 14 and 15.

In a preferred embodiment, XA proteins have variable positions selected solely from 'Active Site Region' residues of *B. circulans* xylanase. Alternatively, at least a majority (51%) of the variable

positions are selected from 'Active Site Region' residues, with at least about 75% of the variable positions being preferably selected from 'Active Site Region' residue positions, and at least about 90% of the variable positions being particularly preferred. A specifically preferred embodiment has only 'Active Site Region' variable positions altered as compared to *B. circulans xylanase*.

- 5 In another preferred embodiment, the XA protein of the invention has a sequence that differs from a wild type *B. circulans* xylanase sequence in at least one amino acid position selected from the positions: 5, 7, 11, 37, 39, 63, 65, 67, 71, 80, 82, 88, 110, 115, 118, 125, 129, 168, and 170, (see Figure 3).

- 10 In one aspect of this embodiment, preferred amino acid changes within the 'Active Site Region' are as follows (see Figure 14A): Y5W; Y5F; Y5H; Q7E; Q7L; D11I; D11V; D11M; D11L; D11E; D11T; D11Q; D11Y; D11F; D11N; D11S; D11A; V37D; V37M; V37S; V37I; V37E; V37A; V37L; G39A; G39S; N63W; N63Q; N63A; N63D; Y65E; Y65L; Y65M; T67E; T67D; T67L; T67A; W71V; W71F; W71M; W71D; W71E; W71I; W71S; Y80M; Y80L; Y80F; Y80I; Y80E; V82D; Y88N; Y88K; Y88W; Y88F; Y88Q; Y88D; Y88M; T110D; A115Y; A115T; A115D; A115S; A115F; I118E; I118D; I118V; I118A;  
15 F125Y; F125M; F125L; W129E; W129S; W129L; W129M; V168D; V168A; A170S; A170T, and A170D. These may be done either individually or in combination, with any combination possible. However, as outlined herein, preferred embodiments utilize at least five, and preferably more variable positions in each XA protein.

- 20 In one particularly preferred aspect of this embodiment, a preferred XA protein comprises the following changes (see Figures 14B): Q7E, G39A, N63W, Y65E, T67E, Y88N, T110D, I118E, W129E, and V168D.

In another preferred embodiment, the substrate is included in the PDA design of the 'Active site Region'.

- 25 In one particularly preferred aspect of this embodiment, a preferred XA protein comprises the following changes (see Figures 15B): G39S, N63W, Y65E, T67E, Y80M, T110D; W129L, V168D, and A170T.

Any of the above described mutations may be done either individually or in combination, with any combination possible. For example, two mutations selected by the PDA design of the CORE may be combined with 4 mutations selected by the PDA design of the 'Helix Region'.

In a preferred embodiment, the XA proteins of the invention are *B. circulans* xylanase conformers. By "conformer" herein is meant a protein that has a protein backbone 3D structure that is virtually the same but has significant differences in the amino acid side chains. That is, the XA proteins of the invention define a conformer set, wherein all of the proteins of the set share a backbone structure and yet have sequences that differ by at least 3-5%. The three dimensional backbone structure of an XA protein thus substantially corresponds to the three dimensional backbone structure of *B. circulans* xylanase. "Backbone" in this context means the non-side chain atoms: the nitrogen, carbonyl carbon and oxygen, and the  $\alpha$ -carbon, and the hydrogens attached to the nitrogen and  $\alpha$ -carbon. To be considered a conformer, a protein must have backbone atoms that are no more than 2 Å from the *B. circulans* xylanase structure, with no more than 1.5 Å being preferred, and no more than 1 Å being particularly preferred. In general, these distances may be determined in two ways. In one embodiment, each potential conformer is crystallized and its three dimensional structure determined. Alternatively, as the former is quite tedious, the sequence of each potential conformer is run in the PDA program to determine whether it is a conformer.

XA proteins may also be identified as being encoded by XA nucleic acids. In the case of the nucleic acid, the overall homology of the nucleic acid sequence is commensurate with amino acid homology but takes into account the degeneracy in the genetic code and codon bias of different organisms. Accordingly, the nucleic acid sequence homology may be either lower or higher than that of the protein sequence, with lower homology being preferred.

In a preferred embodiment, an XA nucleic acid encodes an XA protein. As will be appreciated by those in the art, due to the degeneracy of the genetic code, an extremely large number of nucleic acids may be made, all of which encode the XA proteins of the present invention. Thus, having identified a particular amino acid sequence, those skilled in the art could make any number of different nucleic acids, by simply modifying the sequence of one or more codons in a way which does not change the amino acid sequence of the XA.

In one embodiment, the nucleic acid homology is determined through hybridization studies. Thus, for example, nucleic acids which hybridize under high stringency to the nucleic acid sequence shown in Figure 1 or its complement and encode a XA protein is considered an XA gene.

High stringency conditions are known in the art; see for example Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2d Edition, 1989, and *Short Protocols in Molecular Biology*, ed. Ausubel, et al., both of which are hereby incorporated by reference. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher

temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point ( $T_m$ ) for the specific sequence at a defined ionic strength and pH. The  $T_m$  is the temperature (under defined ionic strength, pH and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at  $T_m$ , 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g. 10 to 50 nucleotides) and at least about 60°C for long probes (e.g. greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide.

In another embodiment, less stringent hybridization conditions are used; for example, moderate or low stringency conditions may be used, as are known in the art; see Sambrook et al., supra; Ausubel et al., supra, and Tijssen, supra.

The XA proteins and nucleic acids of the present invention are recombinant. As used herein, "nucleic acid" may refer to either DNA or RNA, or molecules which contain both deoxy- and ribonucleotides. The nucleic acids include genomic DNA, cDNA and oligonucleotides including sense and anti-sense nucleic acids. Such nucleic acids may also contain modifications in the ribose-phosphate backbone to increase stability and half life of such molecules in physiological environments.

The nucleic acid may be double stranded, single stranded, or contain portions of both double stranded or single stranded sequence. As will be appreciated by those in the art, the depiction of a single strand ("Watson") also defines the sequence of the other strand ("Crick"); thus the sequence depicted in Figure 1 also includes the complement of the sequence. By the term "recombinant nucleic acid" herein is meant nucleic acid, originally formed *in vitro*, in general, by the manipulation of nucleic acid by endonucleases, in a form not normally found in nature. Thus an isolated XA nucleic acid, in a linear form, or an expression vector formed *in vitro* by ligating DNA molecules that are not normally joined, are both considered recombinant for the purposes of this invention. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell or organism, it will replicate non-recombinantly, i.e. using the *in vivo* cellular machinery of the host cell rather than *in vitro* manipulations; however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purposes of the invention.



Similarly, a "recombinant protein" is a protein made using recombinant techniques, i.e. through the expression of a recombinant nucleic acid as depicted above. A recombinant protein is distinguished from naturally occurring protein by at least one or more characteristics. For example, the protein may be isolated or purified away from some or all of the proteins and compounds with which it is normally associated in its wild type host, and thus may be substantially pure. For example, an isolated protein is unaccompanied by at least some of the material with which it is normally associated in its natural state, preferably constituting at least about 0.5%, more preferably at least about 5% by weight of the total protein in a given sample. A substantially pure protein comprises at least about 75% by weight of the total protein, with at least about 80% being preferred, and at least about 90% being particularly preferred. The definition includes the production of an XA protein from one organism in a different organism or host cell. Alternatively, the protein may be made at a significantly higher concentration than is normally seen, through the use of an inducible promoter or high expression promoter, such that the protein is made at increased concentration levels. Furthermore, all of the XA proteins outlined herein are in a form not normally found in nature, as they contain amino acid substitutions, insertions and deletions, with substitutions being preferred, as discussed below.

Also included within the definition of XA proteins of the present invention are amino acid sequence variants of the XA sequences outlined herein and shown in the Figures. That is, the XA proteins may contain additional variable positions as compared to wild type xylanase. These variants fall into one or more of three classes: substitutional, insertional or deletional variants. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding an XA protein, using cassette or PCR mutagenesis or other techniques well known in the art, to produce DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture as outlined above. However, variant XA protein fragments having up to about 100-150 residues may be prepared by *in vitro* synthesis using established techniques. Amino acid sequence variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring allelic or interspecies variation of the XA protein amino acid sequence. The variants typically exhibit the same qualitative biological activity as the naturally occurring analogue, although variants can also be selected which have modified characteristics as will be more fully outlined below.

While the site or region for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed XA variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well

known, for example, M13 primer mutagenesis and PCR mutagenesis. Screening of the mutants is done using assays of XA protein activities.

Amino acid substitutions are typically of single residues; insertions usually will be on the order of from about 1 to 20 amino acids, although considerably larger insertions may be tolerated. Deletions range from about 1 to about 20 residues, although in some cases deletions may be much larger.

Substitutions, deletions, insertions or any combination thereof may be used to arrive at a final derivative. Generally these changes are done on a few amino acids to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances. When small alterations in the characteristics of the XA protein are desired, substitutions are generally made in accordance with the following chart:

Chart I	
<u>Original Residue</u>	<u>Exemplary Substitutions</u>
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser, Ala
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those shown in Chart I. For example, substitutions may be made which more significantly affect: the structure of the polypeptide backbone in the area of the alteration, for example the alpha-helical or beta-sheet structure; the charge or hydrophobicity of the molecule at the target site; or the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the polypeptide's properties are those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an

electropositive side chain, e.g. lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g. glycine.

5 The variants typically exhibit the same qualitative biological activity and will elicit the same immune response as the original XA protein, although variants also are selected to modify the characteristics of the XA proteins as needed. Alternatively, the variant may be designed such that the biological activity of the XA protein is altered. For example, glycosylation sites may be altered or removed. Similarly, the biological function may be altered; for example, in some instances it may be desirable to have more or less potent xylanase activity.

10 The XA proteins and nucleic acids of the invention can be made in a number of ways. Individual nucleic acids and proteins can be made as known in the art and outlined below. Alternatively, libraries of XA proteins can be made for testing.

15 In a preferred embodiment, sets or libraries of XA proteins are generated from a probability distribution table. As outlined herein, there are a variety of methods of generating a probability distribution table, including using PDA, sequence alignments, forcefield calculations such as SCMF calculations, etc. In addition, the probability distribution can be used to generate information entropy scores for each position, as a measure of the mutational frequency observed in the library.

20 In this embodiment, the frequency of each amino acid residue at each variable position in the list is identified. Frequencies can be thresholded, wherein any variant frequency lower than a cutoff is set to zero. This cutoff is preferably 1%, 2%, 5%, 10% or 20%, with 10% being particularly preferred. These frequencies are then built into the XA protein library. That is, as above, these variable positions are collected and all possible combinations are generated, but the amino acid residues that "fill" the library are utilized on a frequency basis. Thus, in a non-frequency based library, a variable position that has 5 possible residues will have 20% of the proteins comprising that variable position with the first possible residue, 20% with the second, etc. However, in a frequency based library, a variable position that has 5 possible residues with frequencies of 10%, 15%, 25%, 30% and 20%, respectively, will have 10% of the proteins comprising that variable position with the first possible residue, 15% of the proteins with the second residue, 25% with the third, etc. As will be appreciated by those in the art, the actual frequency may depend on the method used to actually generate the proteins; for example, exact frequencies may be possible when the proteins are synthesized. However, when the frequency-based primer system outlined below is used, the actual frequencies at each position will vary, as outlined below.

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As will be appreciated by those in the art and outlined herein, probability distribution tables can be generated in a variety of ways. In addition to the methods outlined herein, self-consistent mean field (SCMF) methods can be used in the direct generation of probability tables. SCMF is a deterministic computational method that uses a mean field description of rotamer interactions to calculate energies.

5 A probability table generated in this way can be used to create libraries as described herein. SCMF can be used in three ways: the frequencies of amino acids and rotamers for each amino acid are listed at each position; the probabilities are determined directly from SCMF (see Delarue et al. *Pac. Symp. Biocomput.* 109-21 (1997), expressly incorporated by reference). In addition, highly variable positions and non-variable positions can be identified. Alternatively, another method is used to determine what  
10 sequence is jumped to during a search of sequence space; SCMF is used to obtain an accurate energy for that sequence; this energy is then used to rank it and create a rank-ordered list of sequences (similar to a Monte Carlo sequence list). A probability table showing the frequencies of amino acids at each position can then be calculated from this list (Koehl et al., *J. Mol. Biol.* 239:249 (1994); Koehl et al., *Nat. Struc. Biol.* 2:163 (1995); Koehl et al., *Curr. Opin. Struct. Biol.* 6:222 (1996);  
15 Koehl et al., *J. Mol. Bio.* 293:1183 (1999); Koehl et al., *J. Mol. Biol.* 293:1161 (1999); Lee *J. Mol. Biol.* 236:918 (1994); and Vasquez *Biopolymers* 36:53-70 (1995); all of which are expressly incorporated by reference. Similar methods include, but are not limited to, OPLS-AA (Jorgensen, et al., *J. Am. Chem. Soc.* (1996), v 118, pp 11225-11236; Jorgensen, W.L.; BOSS, Version 4.1; Yale University: New Haven, CT (1999)); OPLS (Jorgensen, et al., *J. Am. Chem. Soc.* (1988), v 110, pp 1657ff; Jorgensen,  
20 et al., *J Am. Chem. Soc.* (1990), v 112, pp 4768ff); UNRES (United Residue Forcefield; Liwo, et al., *Protein Science* (1993), v 2, pp1697-1714; Liwo, et al., *Protein Science* (1993), v 2, pp1715-1731; Liwo, et al., *J. Comp. Chem.* (1997), v 18, pp849-873; Liwo, et al., *J. Comp. Chem.* (1997), v 18, pp874-884; Liwo, et al., *J. Comp. Chem.* (1998), v 19, pp259-276; Forcefield for Protein Structure Prediction (Liwo, et al., *Proc. Natl. Acad. Sci. USA* (1999), v 96, pp5482-5485); ECEPP/3 (Liwo et al.,  
25 *J Protein Chem* 1994 May;13(4):375-80); AMBER 1.1 force field (Weiner, et al., *J. Am. Chem. Soc.* v106, pp765-784); AMBER 3.0 force field (U.C. Singh et al., *Proc. Natl. Acad. Sci. USA.* 82:755-759); CHARMM and CHARMM22 (Brooks, et al., *J. Comp. Chem.* v4, pp 187-217); cvff3.0 (Dauber-Osguthorpe, et al., (1988) *Proteins: Structure, Function and Genetics*, v4, pp31-47); cff91 (Maple, et al., *J. Comp. Chem.* v15, 162-182); also, the DISCOVER (cvff and cff91) and AMBER  
30 forcefields are used in the INSIGHT molecular modeling package (Biosym/MSI, San Diego California) and HARMM is used in the QUANTA molecular modeling package (Biosym/MSI, San Diego California).

In addition, as outlined herein, a preferred method of generating a probability distribution table is through the use of sequence alignment programs. In addition, the probability table can be obtained by  
35 a combination of sequence alignments and computational approaches. For example, one can add

amino acids found in the alignment of homologous sequences to the result of the computation. Preferable one can add the wild type amino acid identity to the probability table if it is not found in the computation.

As will be appreciated, an XA protein library created by recombining variable positions and/or residues at the variable position may not be in a rank-ordered list. In some embodiments, the entire list may just be made and tested. Alternatively, in a preferred embodiment, the XA protein library is also in the form of a rank ordered list. This may be done for several reasons, including the size of the library is still too big to generate experimentally, or for predictive purposes. This may be done in several ways. In one embodiment, the library is ranked using the scoring functions of PDA to rank the library members. Alternatively, statistical methods could be used. For example, the library may be ranked by frequency score; that is, proteins containing the most of high frequency residues could be ranked higher, etc. This may be done by adding or multiplying the frequency at each variable position to generate a numerical score. Similarly, the library different positions could be weighted and then the proteins scored; for example, those containing certain residues could be arbitrarily ranked.

In a preferred embodiment, the different protein members of the XA protein library may be chemically synthesized. This is particularly useful when the designed proteins are short, preferably less than 150 amino acids in length, with less than 100 amino acids being preferred, and less than 50 amino acids being particularly preferred, although as is known in the art, longer proteins can be made chemically or enzymatically. See for example Wilken et al, Curr. Opin. Biotechnol. 9:412-26 (1998), hereby expressly incorporated by reference.

In a preferred embodiment, particularly for longer proteins or proteins for which large samples are desired, the library sequences are used to create nucleic acids such as DNA which encode the member sequences and which can then be cloned into host cells, expressed and assayed, if desired. Thus, nucleic acids, and particularly DNA, can be made which encodes each member protein sequence. This is done using well known procedures. The choice of codons, suitable expression vectors and suitable host cells will vary depending on a number of factors, and can be easily optimized as needed.

In a preferred embodiment, multiple PCR reactions with pooled oligonucleotides is done, as is generally depicted in Figure 17. In this embodiment, overlapping oligonucleotides are synthesized which correspond to the full length gene. Again, these oligonucleotides may represent all of the different amino acids at each variant position or subsets.

In a preferred embodiment, these oligonucleotides are pooled in equal proportions and multiple PCR reactions are performed to create full length sequences containing the combinations of mutations defined by the library. In addition, this may be done using error-prone PCR methods.

5 In a preferred embodiment, the different oligonucleotides are added in relative amounts corresponding to the probability distribution table. The multiple PCR reactions thus result in full length sequences with the desired combinations of mutations in the desired proportions.

The total number of oligonucleotides needed is a function of the number of positions being mutated and the number of mutations being considered at these positions:

10 (number of oligos for constant positions) + M1 + M2 + M3 + ... Mn = (total number of oligos required), where Mn is the number of mutations considered at position n in the sequence.

15 In a preferred embodiment, each overlapping oligonucleotide comprises only one position to be varied; in alternate embodiments, the variant positions are too close together to allow this and multiple variants per oligonucleotide are used to allow complete recombination of all the possibilities. That is, each oligo can contain the codon for a single position being mutated, or for more than one position being mutated. The multiple positions being mutated must be close in sequence to prevent the oligo length from being impractical. For multiple mutating positions on an oligonucleotide, particular combinations of mutations can be included or excluded in the library by including or excluding the oligonucleotide encoding that combination. For example, as discussed herein, there may be correlations between variable regions; that is, when position X is a certain residue, position Y must (or must not) be a particular residue. These sets of variable positions are sometimes referred to herein as a "cluster". When the clusters are comprised of residues close together, and thus can reside on one oligonucleotide primer, the clusters can be set to the "good" correlations, and eliminate the bad combinations that may decrease the effectiveness of the library. However, if the residues of the cluster are far apart in sequence, and thus will reside on different oligonucleotides for synthesis, it may be desirable to either set the residues to the "good" correlation, or eliminate them as variable residues entirely. In an alternative embodiment, the library may be generated in several steps, so that the cluster mutations only appear together. This procedure, i.e. the procedure of identifying mutation clusters and either placing them on the same oligonucleotides or eliminating them from the library or library generation in several steps preserving clusters, can considerably enrich the experimental library with properly folded protein. Identification of clusters can be carried out by a number of ways, e.g. by using known pattern recognition methods, comparisons of frequencies of occurrence of mutations or by using energy analysis of the sequences to be experimentally generated (for example, if the energy of interaction is high, the positions are correlated). These correlations may be positional correlations (e.g.

variable positions 1 and 2 always change together or never change together) or sequence correlations (e.g. if there is residue A at position 1, there is always residue B at position 2). See: Pattern discovery in Biomolecular Data: Tools, Techniques, and Applications; edited by Jason T.L. Wang, Bruce A. Shapiro, Dennis Shasha. New York: Oxford University, 1999; Andrews, Harry C. Introduction to mathematical techniques in pattern recognition; New York, Wiley-Interscience [1972]; Applications of Pattern Recognition; Editor, K.S. Fu. Boca Raton, Fla. CRC Press, 1982; Genetic Algorithms for Pattern Recognition; edited by Sankar K. Pal, Paul P. Wang. Boca Raton: CRC Press, c1996; Pandya, Abhijit S., Pattern recognition with neural networks in C++ / Abhijit S. Pandya, Robert B. Macy. Boca Raton, Fla.: CRC Press, 1996; Handbook of pattern recognition & computer vision / edited by C.H. Chen, L.F. Pau, P.S.P. Wang. 2nd ed. Singapore; River Edge, N.J.: World Scientific, c1999; Friedman, Introduction to Pattern Recognition: Statistical, Structural, Neural, and Fuzzy Logic Approaches; River Edge, N.J.: World Scientific, c1999, Series title: Series in machine perception and artificial intelligence; vol. 32; all of which are expressly incorporated by reference. In addition, programs used to search for consensus motifs can be used as well.

In addition, correlations and shuffling can be fixed or optimized by altering the design of the oligonucleotides; that is, by deciding where the oligonucleotides (primers) start and stop (e.g. where the sequences are "cut"). The start and stop sites of oligos can be set to maximize the number of clusters that appear in single oligonucleotides, thereby enriching the library with higher scoring sequences. Different oligonucleotide start and stop site options can be computationally modeled and ranked according to number of clusters that are represented on single oligos, or the percentage of the resulting sequences consistent with the predicted library of sequences.

The total number of oligonucleotides required increases when multiple mutable positions are encoded by a single oligonucleotide. The annealed regions are the ones that remain constant, i.e. have the sequence of the reference sequence.

Oligonucleotides with insertions or deletions of codons can be used to create a library expressing different length proteins. In particular computational sequence screening for insertions or deletions can result in secondary libraries defining different length proteins, which can be expressed by a library of pooled oligonucleotide of different lengths.

In a preferred embodiment, the XA protein library is done by shuffling the family (e.g. a set of variants); that is, some set of the top sequences (if a rank-ordered list is used) can be shuffled, either with or without error-prone PCR. "Shuffling" in this context means a recombination of related sequences, generally in a random way. It can include "shuffling" as defined and exemplified in U.S. Patent Nos.

5,830,721; 5,811,238; 5,605,793; 5,837,458 and PCT US/19256, all of which are expressly incorporated by reference in their entirety. This set of sequences can also be an artificial set; for example, from a probability table (for example generated using SCMF) or a Monte Carlo set. Similarly, the "family" can be the top 10 and the bottom 10 sequences, the top 100 sequence, etc. This may also be done using error-prone PCR.

Thus, in a preferred embodiment, in silico shuffling is done using the computational methods described herein. That is, starting with either two libraries or two sequences, random recombinations of the sequences can be generated and evaluated.

In a preferred embodiment, error-prone PCR is done to generate the XA protein library. See U.S. Patent Nos. 5,605,793, 5,811,238, and 5,830,721, all of which are hereby incorporated by reference. This can be done on the optimal sequence or on top members of the library, or some other artificial set or family. In this embodiment, the gene for the optimal sequence found in the computational screen of the primary library can be synthesized. Error prone PCR is then performed on the optimal sequence gene in the presence of oligonucleotides that code for the mutations at the variant positions of the library (bias oligonucleotides). The addition of the oligonucleotides will create a bias favoring the incorporation of the mutations in the library. Alternatively, only oligonucleotides for certain mutations may be used to bias the library.

In a preferred embodiment, gene shuffling with error prone PCR can be performed on the gene for the optimal sequence, in the presence of bias oligonucleotides, to create a DNA sequence library that reflects the proportion of the mutations found in the XA protein library. The choice of the bias oligonucleotides can be done in a variety of ways; they can be chosen on the basis of their frequency, i.e. oligonucleotides encoding high mutational frequency positions can be used; alternatively, oligonucleotides containing the most variable positions can be used, such that the diversity is increased; if the secondary library is ranked, some number of top scoring positions can be used to generate bias oligonucleotides; random positions may be chosen; a few top scoring and a few low scoring ones may be chosen; etc. What is important is to generate new sequences based on preferred variable positions and sequences.

In a preferred embodiment, PCR using a wild type gene or other gene can be used, as is schematically depicted in Figure 18. In this embodiment, a starting gene is used; generally, although this is not required, the gene is usually the wild type gene. In some cases it may be the gene encoding the global optimized sequence, or any other sequence of the list, or a consensus sequence obtained e.g. from aligning homologous sequences from different organisms. In this embodiment,



oligonucleotides are used that correspond to the variant positions and contain the different amino acids of the library. PCR is done using PCR primers at the termini, as is known in the art. This provides two benefits; the first is that this generally requires fewer oligonucleotides and can result in fewer errors. In addition, it has experimental advantages in that if the wild type gene is used, it need not be synthesized.

In addition, there are several other techniques that can be used, as exemplified in the figures, e.g. Figures 19-21. In a preferred embodiment, ligation of PCR products is done.

In a preferred embodiment, a variety of additional steps may be done to the XA protein library; for example, further computational processing can occur, different XA libraries can be recombined, or cutoffs from different libraries can be combined. In a preferred embodiment, an XA protein library may be computationally remanipulated to form an additional XA protein library (sometimes referred to herein as "tertiary libraries"). For example, any of the XA protein library sequences may be chosen for a second round of PDA, by freezing or fixing some or all of the changed positions in the first library. Alternatively, only changes seen in the last probability distribution table are allowed. Alternatively, the stringency of the probability table may be altered, either by increasing or decreasing the cutoff for inclusion. Similarly, the XA protein library may be recombined experimentally after the first round; for example, the best gene/genes from the first screen may be taken and gene assembly redone (using techniques outlined below, multiple PCR, error prone PCR, shuffling, etc.). Alternatively, the fragments from one or more good gene(s) to change probabilities at some positions. This biases the search to an area of sequence space found in the first round of computational and experimental screening.

In a preferred embodiment, a tertiary library can be generated from combining different XA libraries. For example, a probability distribution table from a first XA protein library can be generated and recombined, either computationally or experimentally, as outlined herein. A PDA XA protein library may be combined with a sequence alignment XA protein library, and either recombined (again, computationally or experimentally) or just the cutoffs from each joined to make a new tertiary library. The top sequences from several libraries can be recombined. Sequences from the top of a library can be combined with sequences from the bottom of the library to more broadly sample sequence space, or only sequences distant from the top of the library can be combined. XA libraries that analyzed different parts of a protein can be combined to a tertiary library that treats the combined parts of the protein.

In a preferred embodiment, a tertiary library can be generated using correlations in an XA protein library. That is, a residue at a first variable position may be correlated to a residue at second variable

position (or correlated to residues at additional positions as well). For example, two variable positions may sterically or electrostatically interact, such that if the first residue is X, the second residue must be Y. This may be either a positive or negative correlation.

5 Using the nucleic acids of the present invention which encode an XA protein, a variety of expression vectors are made. The expression vectors may be either self-replicating extrachromosomal vectors or vectors which integrate into a host genome. Generally, these expression vectors include transcriptional and translational regulatory nucleic acid operably linked to the nucleic acid encoding the XA protein. The term "control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. The control sequences that are  
10 suitable for prokaryotes, for example, include a promoter, optionally an operator sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers.

15 Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation.

20 In a preferred embodiment, when the endogenous secretory sequence leads to a low level of secretion of the naturally occurring protein or of the XA protein, a replacement of the naturally occurring secretory leader sequence is desired. In this embodiment, an unrelated secretory leader sequence is operably linked to an XA encoding nucleic acid leading to increased protein secretion. Thus, any secretory leader sequence resulting in enhanced secretion of the XA protein, when compared to the  
25 secretion of the wild type xylanase and its secretory sequence, is desired. Suitable secretory leader sequences that lead to the secretion of a protein are known in the art.

In another preferred embodiment, a secretory leader sequence of a naturally occurring protein or a protein is removed by techniques known in the art and subsequent expression results in intracellular accumulation of the recombinant protein.

30 Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not

exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice. The transcriptional and translational regulatory nucleic acid will generally be appropriate to the host cell used to express the fusion protein; for example, transcriptional and translational regulatory nucleic acid sequences from *Bacillus* are preferably used to express the fusion protein in *Bacillus*. Numerous types of appropriate expression vectors, and suitable regulatory sequences are known in the art for a variety of host cells.

In general, the transcriptional and translational regulatory sequences may include, but are not limited to, promoter sequences, ribosomal binding sites, transcriptional start and stop sequences, translational start and stop sequences, and enhancer or activator sequences. In a preferred embodiment, the regulatory sequences include a promoter and transcriptional start and stop sequences.

Promoter sequences encode either constitutive or inducible promoters. The promoters may be either naturally occurring promoters or hybrid promoters. Hybrid promoters, which combine elements of more than one promoter, are also known in the art, and are useful in the present invention. In a preferred embodiment, the promoters are strong promoters, allowing high expression in cells, particularly mammalian cells, such as the CMV promoter, particularly in combination with a Tet regulatory element.

In addition, the expression vector may comprise additional elements. For example, the expression vector may have two replication systems, thus allowing it to be maintained in two organisms, for example in mammalian or insect cells for expression and in a prokaryotic host for cloning and amplification. Furthermore, for integrating expression vectors, the expression vector contains at least one sequence homologous to the host cell genome, and preferably two homologous sequences which flank the expression construct. The integrating vector may be directed to a specific locus in the host cell by selecting the appropriate homologous sequence for inclusion in the vector. Constructs for integrating vectors are well known in the art.

In addition, in a preferred embodiment, the expression vector contains a selectable marker gene to allow the selection of transformed host cells. Selection genes are well known in the art and will vary with the host cell used.

A preferred expression vector system is a retroviral vector system such as is generally described in PCT/US97/01019 and PCT/US97/01048, both of which are hereby expressly incorporated by reference.

The XA nucleic acids are introduced into the cells either alone or in combination with an expression vector. By "introduced into" or grammatical equivalents herein is meant that the nucleic acids enter the cells in a manner suitable for subsequent expression of the nucleic acid. The method of introduction is largely dictated by the targeted cell type, discussed below. Exemplary methods include (Ca<sub>3</sub>PO<sub>4</sub>)<sub>2</sub> precipitation, liposome fusion, lipofectin®, electroporation, viral infection, etc. The XA nucleic acids may stably integrate into the genome of the host cell (for example, with retroviral introduction, outlined below), or may exist either transiently or stably in the cytoplasm (i.e. through the use of traditional plasmids, utilizing standard regulatory sequences, selection markers, etc.).

The XA proteins of the present invention are produced by culturing a host cell transformed with an expression vector containing nucleic acid encoding an XA protein, under the appropriate conditions to induce or cause expression of the XA protein. The conditions appropriate for XA protein expression will vary with the choice of the expression vector and the host cell, and will be easily ascertained by one skilled in the art through routine experimentation. For example, the use of constitutive promoters in the expression vector will require optimizing the growth and proliferation of the host cell, while the use of an inducible promoter requires the appropriate growth conditions for induction. In addition, in some embodiments, the timing of the harvest is important. For example, the baculoviral systems used in insect cell expression are lytic viruses, and thus harvest time selection can be crucial for product yield.

Appropriate host cells include yeast, bacteria, archaeobacteria, fungi, and insect and animal cells, including mammalian cells. Of particular interest are *Drosophila melanogaster* cells, *Saccharomyces cerevisiae* and other yeasts, *E. coli*, *Bacillus subtilis*, SF9 cells, C129 cells, 293 cells, *Neurospora*, BHK, CHO, COS, *Pichia Pastoris*, *Aspergillus*, *Trichoderma*, etc.

In a preferred embodiment, XA proteins of the invention are expressed in filamentous fungi. Filamentous fungi are eukaryotic microorganisms and include all filamentous forms of the subdivision Eumycotina. Various species of filamentous fungi may be used as expression hosts, including the following genera: *Aspergillus*, *Trichoderma*, *Neurospora*, *Podospora*, *Endothia Mucor*, *Cochiobolus* and *Pyricularia*. Specific expression hosts include *A. nidulans*, *A. niger*, *A. awomari*, *A. oryzae*, *N. crassa*, *Trichoderma reesei*, and *Trichoderma viride*. Suitable vectors and methods for expression and secretion of heterologous polypeptides from filamentous fungi are described in US Patent No. 6,004,785, hereby expressly incorporated by reference.

In a preferred embodiment, the XA proteins are expressed in mammalian cells. Mammalian expression systems are also known in the art, and include retroviral systems. A mammalian promoter

is any DNA sequence capable of binding mammalian RNA polymerase and initiating the downstream (3') transcription of a coding sequence for the fusion protein into mRNA. A promoter will have a transcription initiating region, which is usually placed proximal to the 5' end of the coding sequence, and a TATA box, using a located 25-30 base pairs upstream of the transcription initiation site. The TATA box is thought to direct RNA polymerase II to begin RNA synthesis at the correct site. A mammalian promoter will also contain an upstream promoter element (enhancer element), typically located within 100 to 200 base pairs upstream of the TATA box. An upstream promoter element determines the rate at which transcription is initiated and can act in either orientation. Of particular use as mammalian promoters are the promoters from mammalian viral genes, since the viral genes are often highly expressed and have a broad host range. Examples include the SV40 early promoter, mouse mammary tumor virus LTR promoter, adenovirus major late promoter, herpes simplex virus promoter, and the CMV promoter.

Typically, transcription termination and polyadenylation sequences recognized by mammalian cells are regulatory regions located 3' to the translation stop codon and thus, together with the promoter elements, flank the coding sequence. The 3' terminus of the mature mRNA is formed by site-specific post-translational cleavage and polyadenylation. Examples of transcription terminator and polyadenylation signals include those derived from SV40.

The methods of introducing exogenous nucleic acid into mammalian hosts, as well as other hosts, is well known in the art, and will vary with the host cell used. Techniques include dextran-mediated transfection, calcium phosphate precipitation, polybrene mediated transfection, protoplast fusion, electroporation, viral infection, encapsulation of the polynucleotide(s) in liposomes, and direct microinjection of the DNA into nuclei. As outlined herein, a particularly preferred method utilizes retroviral infection, as outlined in PCT US97/01019, incorporated by reference.

As will be appreciated by those in the art, the type of mammalian cells used in the present invention can vary widely. Basically, any mammalian cells may be used, with mouse, rat, primate and human cells being particularly preferred. Accordingly, suitable cell types include, but are not limited to, tumor cells of all types (particularly melanoma, myeloid leukemia, carcinomas of the lung, breast, ovaries, colon, kidney, prostate, pancreas and testes), cardiomyocytes, endothelial cells, epithelial cells, lymphocytes (T-cell and B cell), mast cells, eosinophils, vascular intimal cells, hepatocytes, leukocytes including mononuclear leukocytes, stem cells such as hematopoietic, neural, skin, lung, kidney, liver and myocyte stem cells (for use in screening for differentiation and de-differentiation factors), osteoclasts, chondrocytes and other connective tissue cells, keratinocytes, melanocytes, liver cells, kidney cells, and adipocytes. Suitable cells also include known research cells, including, but not

limited to, Jurkat T cells, NIH3T3 cells, CHO, Cos, etc. See the ATCC cell line catalog, hereby expressly incorporated by reference.

In one embodiment, the cells may be additionally genetically engineered, that is, contain exogenous nucleic acid other than the XA nucleic acid.

- 5 In a preferred embodiment, the XA proteins are expressed in bacterial systems. Bacterial expression systems are well known in the art. Sung et al. [Protein Expression and Purification 4:200-206 (1993); hereby expressly incorporated by reference] report expression of B.circulans in *E.coli*.

A suitable bacterial promoter is any nucleic acid sequence capable of binding bacterial RNA  
polymerase and initiating the downstream (3') transcription of the coding sequence of the XA protein  
10 into mRNA. A bacterial promoter has a transcription initiation region which is usually placed proximal to the 5' end of the coding sequence. This transcription initiation region typically includes an RNA polymerase binding site and a transcription initiation site. Sequences encoding metabolic pathway enzymes provide particularly useful promoter sequences. Examples include promoter sequences  
15 derived from sugar metabolizing enzymes, such as galactose, lactose and maltose, and sequences derived from biosynthetic enzymes such as tryptophan. Promoters from bacteriophage may also be used and are known in the art. In addition, synthetic promoters and hybrid promoters are also useful; for example, the *tac* promoter is a hybrid of the *trp* and *lac* promoter sequences. Furthermore, a bacterial promoter can include naturally occurring promoters of non-bacterial origin that have the ability to bind bacterial RNA polymerase and initiate transcription.

20

In addition to a functioning promoter sequence, an efficient ribosome binding site is desirable. In *E. coli*, the ribosome binding site is called the Shine-Delgarno (SD) sequence and includes an initiation codon and a sequence 3-9 nucleotides in length located 3 - 11 nucleotides upstream of the initiation codon.

- 25 The expression vector may also include a signal peptide sequence that provides for secretion of the XA protein in bacteria. The signal sequence typically encodes a signal peptide comprised of hydrophobic amino acids which direct the secretion of the protein from the cell, as is well known in the art. The protein is either secreted into the growth media (gram-positive bacteria) or into the periplasmic space, located between the inner and outer membrane of the cell (gram-negative  
30 bacteria). For expression in bacteria, usually bacterial secretory leader sequences, operably linked to an XA encoding nucleic acid, are preferred.

The bacterial expression vector may also include a selectable marker gene to allow for the selection of bacterial strains that have been transformed. Suitable selection genes include genes which render the bacteria resistant to drugs such as ampicillin, chloramphenicol, erythromycin, kanamycin, neomycin and tetracycline. Selectable markers also include biosynthetic genes, such as those in the histidine, tryptophan and leucine biosynthetic pathways.

These components are assembled into expression vectors. Expression vectors for bacteria are well known in the art, and include vectors for *Bacillus subtilis*, *E. coli*, *Streptococcus cremoris*, and *Streptococcus lividans*, among others.

The bacterial expression vectors are transformed into bacterial host cells using techniques well known in the art, such as calcium chloride treatment, electroporation, and others.

In one embodiment, XA proteins are produced in insect cells. Expression vectors for the transformation of insect cells, and in particular, baculovirus-based expression vectors, are well known in the art.

In a preferred embodiment, XA protein is produced in yeast cells. Yeast expression systems are well known in the art, and include expression vectors for *Saccharomyces cerevisiae*, *Candida albicans* and *C. maltosa*, *Hansenula polymorpha*, *Kluyveromyces fragilis* and *K. lactis*, *Pichia guillermondii* and *P. pastoris*, *Schizosaccharomyces pombe*, and *Yarrowia lipolytica*. Preferred promoter sequences for expression in yeast include the inducible GAL1,10 promoter, the promoters from alcohol dehydrogenase, enolase, glucokinase, glucose-6-phosphate isomerase, glyceraldehyde-3-phosphate-dehydrogenase, hexokinase, phosphofructokinase, 3-phosphoglycerate mutase, pyruvate kinase, and the acid phosphatase gene. Yeast selectable markers include ADE2, HIS4, LEU2, TRP1, and ALG7, which confers resistance to tunicamycin; the neomycin phosphotransferase gene, which confers resistance to G418; and the CUP1 gene, which allows yeast to grow in the presence of copper ions.

In addition, the XA polypeptides of the invention may be further fused to other proteins, if desired, for example to increase expression or stabilize the protein.

Once made, the XA proteins may be covalently modified. One type of covalent modification includes reacting targeted amino acid residues of an XA polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N-or C-terminal residues of an XA polypeptide. Derivatization with bifunctional agents is useful, for instance, for crosslinking an XA protein to a water-insoluble support matrix or surface for use in the method for purifying anti-XA antibodies or screening

assays, as is more fully described below. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)dithio]propioimide.

Other modifications include deamidation of glutamyl and asparagyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the  $\alpha$ -amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the XA polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence XA polypeptide, and/or adding one or more glycosylation sites that are not present in the native sequence XA polypeptide.

Addition of glycosylation sites to XA polypeptides may be accomplished by altering the amino acid sequence thereof. The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the native sequence XA polypeptide (for O-linked glycosylation sites). The XA amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the XA polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids.

Another means of increasing the number of carbohydrate moieties on the XA polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, e.g., in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, *CRC Crit. Rev. Biochem.*, pp. 259-306 (1981).

Removal of carbohydrate moieties present on the XA polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, et al., *Arch. Biochem. Biophys.*, 259:52 (1987) and by Edge et al., *Anal. Biochem.*, 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be



achieved by the use of a variety of endo-and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Another type of covalent modification of XA comprises linking the XA polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

XA polypeptides of the present invention may also be modified in a way to form chimeric molecules comprising an XA polypeptide fused to another, heterologous polypeptide or amino acid sequence. In one embodiment, such a chimeric molecule comprises a fusion of an XA polypeptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino-or carboxyl-terminus of the XA polypeptide. The presence of such epitope-tagged forms of an XA polypeptide can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the XA polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol. 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science 255:192-194 (1992)]; tubulin epitope peptide [Skinner et al., J. Biol. Chem. 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. U.S.A. 87:6393-6397 (1990)].

In a preferred embodiment, the XA protein is purified or isolated after expression. XA proteins may be isolated or purified in a variety of ways known to those skilled in the art depending on what other components are present in the sample. Standard purification methods include electrophoretic, molecular, immunological and chromatographic techniques, including ion exchange, hydrophobic, affinity, and reverse-phase HPLC chromatography, and chromatofocusing. For example, the XA protein may be purified using a standard anti-library antibody column. Ultrafiltration and diafiltration techniques, in conjunction with protein concentration, are also useful. For general guidance in suitable

purification techniques, see Scopes, R., Protein Purification, Springer-Verlag, NY (1982). The degree of purification necessary will vary depending on the use of the XA protein. In some instances no purification will be necessary.

5 Once made, the XA proteins and nucleic acids of the invention find use in a number of applications. In a preferred embodiment, the XA proteins are used in pulp bleaching.

In another preferred embodiment, the XA proteins and nucleic acids of the invention are useful in the bioconversion of lignocellulosic materials to fuels.

10 The XA proteins and nucleic acids of the invention are also useful for clarifying juice and wine [Zeikus et al., ACS Symp. Ser. 460:36-51 (1991); Beily, ACS Symp. Ser. 460:408-416 (1991); Woodward, Top Enzyme Ferment. Biotechnol. 8:9-30 (1984)]; for extracting coffee, plant oils and starch [Beily, supra; Woodward supra; McCleary, Int. J. Biol. Macromol. 8:349-354 (1986)]; for the production of food thickeners (Zeikus et al., supra); for altering texture in bakery products, e.g., improving the quality of dough, help bread rise and processing of wheat and corn for starch production (Maat et al. *In Xylans and xylanases*, eds. Visser et al., Elsevier Sci pub., Amsterdam. ISBN 0-444-894-772 (1992);  
15 McCleary, supra; Krishnarau et al., J. Food Sci. 59:1251-1254 (1994); US Patent No. 5,306,633); for use as animal food additives to aid in the digestibility of feedstuffs; and in the washing of super precision devices and semiconductors [Takayuki et al., U.S. Pat. No. 5,078,802].

In a preferred embodiment, the invention provides a bleaching agent comprising as an active ingredient an XA proteins of the invention.

20 The invention further provides methods for bleaching pulp. This method comprises the step of contacting the pulp with a bleaching agent as described herein. In addition to enzymatic treatment using the bleaching agent comprising an XA protein of the invention, the pulp may be bleached as is known in the art by chemicals and/or alkaline extraction before, after or during the enzymatic treatment. Bleaching chemicals are known in the art and include, but are not limited to chlorine,  
25 chlorine dioxide, nitrogen dioxide, hypochlorites, hydrogen, oxygen peroxide, ozone, etc.

The following examples serve to more fully describe the manner of using the above-described invention, as well as to set forth the best modes contemplated for carrying out various aspects of the invention. It is understood that these examples in no way serve to limit the true scope of this invention, but rather are presented for illustrative purposes. All references cited herein are incorporated by  
30 reference in their entirety.

## EXAMPLE 1

Design and Characterization of Novel XA Proteins by PDA

Summary: Sequences for novel thermostable, thermophilic and/or alkaliphilic xylanase activity (XA) proteins were designed by optimizing (i) residues in the core of the protein, (ii) residues around D83, (iii) residues around the helix region, and (iv) residues around the active site region using Protein Design Automation (PDA) as described in WO98/47089, U.S.S.Nos. 09/058,459, 09/127,926, 60/104,612, 60/158,700, 09/419,351, 60/181,630, 60/186,904, 60/132,475, 60/133,714, U.S patent application, entitled *Protein Design Automation For Protein Libraries* (Filed: April 14, 2000; Inventor: Bassil Dahiyat), and PCT US98/07254, all of which are expressly incorporated by reference in their entirety. Several core designs were completed, with 15-33 residues considered corresponding to  $20^{15}$  –  $20^{33}$  sequence possibilities. Residues unexposed to solvent were designed in order to minimize changes to the molecular surface and to limit the potential for antigenicity of designed novel protein analogues.

Calculations required from 12-67 hours on 16 Silicon Graphics R10000 CPU's. The global optimum sequence from each design was selected for characterization. From 3-27 residues were changed from *Bacillus circulans* xylanase in the designed proteins, out of 185 residues total (mature protein).

COMPUTATIONAL PROTOCOLSTemplate structure preparation:

For this study the crystal structure of *Bacillus circulans* xylanase as deposited in the PDB data bank was used [PDB record 1XNB; Campbell et al., in Suominen and Geinikainen, eds. *Proceedings of the second TRICEL symposium on Trichoderma reesei cellulases and other hydrolases*, Espoo, Finland, Helsinki: Foundation for Biotechnological and Industrial Fermentation Research, pp 63-77 (1993)]. For the PDA design with the substrate, the PDA structure 1BCX was used [Wakarchuk et al., *Protein Sci.* 3:467-475 (1994)]. In the PDA design the mutated position E172C of the PDB structure was forced back to be the wild type glutamate. After removing all water molecules and the sulfate anion and adding all hydrogen atoms to the protein, this resulting structure was minimized for 50 steps with a conjugate gradient method within the Dreiding forcefield without using coulombic forces.

Design strategies:

Core residues, residues around D83, residues around the helix region, and residues around the active site region were selected for design since optimization of these positions can improve stability, although stabilization has been obtained from modifications at other sites as well. Thus, the complete *Bacillus circulans* xylanase was divided into three different regions called 'CORE', 'D83 Region', 'Helix Region', and 'Active Site' by visually inspecting the three dimensional structure.

PDA calculations were run on (i) one set of selected core positions, (ii) two sets of selected 'D83 Region' positions with a total of 6 different designs, (iii) on two sets of selected 'Helix region' positions with a total of 3 different designs, (iv) one set of 'Active Site' positions with a total of 2 designs, one with and one without a substrate (see Figure 3) with the purpose to create a thermostable, thermophilic, and/or alkaliphilic xylanase.

#### PDA calculations

All PDA calculations were performed with solvation model 2. Solvation model 2 is the solvation model described by Street and Mayo [Fold. Design 3:253-258 (1998)]. If possible, Dead End Elimination (DEE) was run to completion to find the PDA ground state. This was done for the PDA calculations for the CORE, the '83 Region' the 'Helix Region', and the 'Active Site', as defined below.

The DEE calculation was for all the given PDA calculation followed by Monte Carlo (MC) minimization and a list of the 1000 lowest energy sequences was generated. In the case of the 'Active Site' ddesign, a Monte Carlo list of 10,000 sequences was generated.

The PDA calculations for all the designs were run using the a2h1p0 rotamer library. This library is based on the backbone-dependent rotamer library of Dunbrack and Karplus (Dunbrack and Karplus, J. Mol. Biol. 230(2):543-74 (1993); hereby expressly incorporated by reference) but includes more rotamers for the aromatic and hydrophobic amino acids;  $\chi_1$  and  $\chi_2$  angle values of rotamers for all the aromatic amino acids and  $\chi_1$  angle values for all the other hydrophobic amino acids were expanded  $\pm 1$  standard deviation about the mean value reported in the Dunbrack and Karplus library. Unless explicitly stated, typical PDA parameters were used: the van der Waals scale factor was set to 0.9, the H-bond potential well-depth was set to 8.0 kcal/mol, the solvation potential was calculated using type 2 solvation with a nonpolar burial energy of 0.048 kcal/mol and a nonpolar exposure multiplication factor of 1.6, and the secondary structure scale factor was set to 0.0 (secondary structure propensities were not considered). Calculations required from 12-67 hours on 16 Silicon Graphics R10000 CPU's.

#### Monte Carlo analysis

Monte Carlo analysis of the sequences produced by PDA calculations shows the ground state (optimal) amino acid and amino acids allowed for each variable position and their frequencies of occurrence (see Figures 4 through 15).

## EXAMPLE 2

### 5                    PDA Calculations for the *Bacillus circulans* Xylanase CORE Region

By visual inspection, the following residues were identified as belonging to the CORE of *Bacillus circulans* xylanase: Y26, V28, W30, F36, V38, I51, Y53, A55, W58, G62, G64, L66, L68, G70, T72, 177, Y79, V81, Y105, I107, S130, A142, I144, F146, W153, M169, T171, G173, S176, G178, S180, V182, and V184 (see Figure 2A).

10            Thus, the following positions were included in the PDA design (see also Figure 3):

```

26  28  30  36  38  51  53  55  58  62  64  66  68  70  72  77  79  81 105 107
Tyr Val Trp Phe Val Ile Tyr Ala Trp Gly Gly Leu Leu Gly Thr Ile Tyr Val Tyr Ile

130 142 144 146 153 169 171 173 176 178 180 182 184
Ser Ala Ile Phe Trp Met Thr Gly Ser Gly Ser Val Val

```

15            A rotamer group was assigned to each CORE position which allows this position to become any phobic residue with the exception of methionine (i.e., Ala, Val, Leu, Ile, Phe, Tyr, and Trp) plus the original wild type residue. Thus, e.g., the group PHOB\_NO\_MET+G was assigned to G62, PHOB\_NO\_MET+T to T72, and PHOB\_NO\_MET+M to M169.

20            In the following PDA design only the CORE residues were allowed to mutate to any amino acid rotamer restricted by the definition of the rotamer group assigned to this position. The rest of the protein was treated as a template, with fixed coordinates. An energy cutoff of 50 kcal/mol for the rotamer/template energy was used to exclude unfavorable rotamers. The van der Waals radius was scaled by a factor of 0.9 and the solvation model 2 as defined by Street and Mayo (supra) was used. The ground state rotamer sequence was extracted from all the possible rotamer sequences using the

25            Dead End Elimination (DEE) method. To check for other low energy sequences a Monte Carlo (MC) search was performed starting from the DEE ground state.

The PDA calculation resulted in the following ground state sequence:

26 28 30 36 38 51 53 55 58 62 64 66 68 70 72 77 79 81 105 107  
Phe Ile Phe Phe Val Ile Phe Ala Phe Gly Val Leu Leu Gly Thr Ile Phe Val Phe Ile  
130 142 144 146 153 169 171 173 176 178 180 182 184  
Ser Leu Ile Phe Trp Met Leu Gly Ala Gly Ala Ile Val

5 This sequence shows 13 mutations when compared to the wild type *Bacillus circulans* xylanase sequence, Y26F, V28I, W30F, Y53F, W58F, G64V, Y79F, Y105F, A142L, T171L, S176A, S180A, and V182I (see Figure 4B). This state shows 93% identity with the complete wild type *Bacillus circulans* xylanase sequence and has 60% identity in the designed positions with the wild type sequence.

10 Using the Monte Carlo technique a list of low energy sequences was generated. The analysis of the lowest 1000 protein sequences generated by Monte Carlo leads to the mutation pattern shown in Figure 4A. Out of the lowest 1000 sequences none has more than 20 mutations from the wild type sequence and out of the lowest 101 sequences none has more than 18 mutations. Thus, any protein sequence showing mutations at the positions according to Figure 4A will potentially generate a more stable and/or active XA protein. In particular those protein sequences found among the list of the  
15 lowest 101 MC generated sequences (data not shown) have a high potential to result in a more stable and active XA protein. A preferred XA sequence is shown in Figure 4B. A DNA library can be generated to mirror the probability table of Figure 4A that comprises at least one sequence that is more stable and/or active than wild type *Bacillus circulans* xylanase.

### EXAMPLE 3

#### 20 PDA Calculations for the *Bacillus circulans* Xylanase Region around D83 (a)

Buried polar residues are known targets that create more stable proteins when mutated to hydrophobic residues as has been shown for the arc repressor protein [Hendsch et al., Biochemistry 35:7621-7625 (1996)]. D83 in *Bacillus circulans* xylanase is such a buried polar residue. PDA was used to design the region around this position (see Figure 2B). All amino acid residues having heavy  
25 side chain atoms within a distance of 7 Å from any heavy side chain atom of D83 that is: Y53, L66, T67, L68, V81, V82, D83, S84, W85, D101, Y105, R132, R136, T138, A142, I144, H149, and M169, were included in the calculation. Of these residues Y53, L66, L68, V81, Y105, A142, I144, H149, and M169, were defined as CORE positions and T67, V82, D83, S84, W85, D101, R132, R136, T138 as BOUNDARY positions. A rotamer group consisting of the phobic residues without methionine (Ala,  
30 Val, Leu, Ile, Phe, Tyr, Trp) plus the wild type amino acid was assigned to the CORE positions. To

D83, R132, and R136 the boundary rotamers (Ala, Val, Leu, Ile, Phe, Trp, Asp, Asn, Glu, Gln, Lys, Ser, Thr, His, Arg, Met) were assigned and to T67, V82, S84, W85, D101, T138 THE BOUNDARY ROTAMERS WITHOUT METHIONINE. The rest of the protein was treated as a fixed template. In the PDA calculation an energy cutoff of 50 kcal/mol was used for the rotamer/template energy to exclude unfavorable rotamers. The van der Waals radius was scaled by a factor of 0.9 and the solvation model 2 as defined by Street and Mayo (supra) was used. The ground state rotamer sequence was extracted from all the possible rotamer sequences using the Dead End Elimination (DEE) method. To check for other low energy sequences a Monte Carlo (MC) search was performed starting from the DEE ground state.

Thus, the following positions were included in the PDA design (see also Figure 3):

53 66 67 68 81 82 83 84 85 101 105 132 136 138 142 144 149 169  
Tyr Leu Thr Leu Val Val Asp Ser Trp Asp Tyr Arg Arg Thr Ala Ile His Met

The PDA calculation resulted in the following ground state sequence:

53 66 67 68 81 82 83 84 85 101 105 132 136 138 142 144 149 169  
Phe Leu Thr Leu Val Val Val Val Phe Asp Phe Met Met Thr Leu Leu Phe Met

This sequence shows 10 mutations when compared to the wild type *Bacillus circulans* xylanase sequence, Y53F, D83V, S84V, W85F, Y105F, R132M, R136M, A142L, I144L, and H149F (see Figure 5B). This DEE ground state shows 95% identity with the complete wild type *Bacillus circulans* xylanase sequence and has 44% identity in the designed positions with the wild type sequence.

Using the Monte Carlo technique a list of low energy sequences was generated. The analysis of the lowest 1000 protein sequences generated by Monte Carlo leads to the mutation pattern shown in Figure 5A. Out of the lowest 1000 sequences none has more than 14 mutations from the wild type sequence and out of the lowest 101 sequences none has more than 13 mutations. Thus, any protein sequence showing mutations at the positions according to Figure 5A will potentially generate a more stable and/or active XA protein. In particular those protein sequences found among the list of the lowest 101 MC generated sequences (data not shown) have a high potential to result in a more stable and active XA protein. A preferred XA sequence is shown in Figure 5B. A DNA library can be generated to mirror the probability table of Figure 5A that comprises at least one sequence that is more stable and/or active than wild type *Bacillus circulans* xylanase.

PDA Calculations for the *Bacillus circulans* Xylanase Region around D83 (b)

A similar calculation as above was performed where D83 was considered a CORE residue, but was still allowed to change its identity to all rotamers of the boundary group.

The PDA calculation resulted in the following ground state sequence, which is identical to the one obtained above in (a):

53 66 67 68 81 82 83 84 85 101 105 132 136 138 142 144 149 169  
Phe Leu Thr Leu Val Val Val Val Phe Asp Phe Met Met Thr Leu Leu Phe Met

Using the Monte Carlo technique a list of low energy sequences was generated. The analysis of the lowest 1000 protein sequences generated by Monte Carlo leads to the mutation pattern shown in Figure 6A. This pattern is slightly different from the one shown in Figure 5A. Out of the lowest 1000 sequences none has more than 13 mutations from the wild type sequence and out of the lowest 101 sequences none has more than 13 mutations. Thus, any protein sequence showing mutations at the positions according to Figure 6A will potentially generate a more stable and/or active XA protein. In particular those protein sequences found among the list of the lowest 101 MC generated sequences (data not shown) have a high potential to result in a more stable and/or active XA protein. A preferred XA sequence is shown in Figure 6B. A DNA library can be generated to mirror the probability table of Figure 6A that comprises at least one sequence that is more stable and/or active than wild type *Bacillus circulans* xylanase.

PDA Calculations for the *Bacillus circulans* Xylanase Region around D83 (c)

A similar calculation as above was performed where T67, V82, W85, which have side chains pointing away from the D83 side chain, were excluded and D83 was considered as a BOUNDARY residue (see Figure 3).

The PDA calculation resulted in the following ground state sequence:

53 66 68 81 83 84 101 105 132 136 138 142 144 149 169  
Phe Leu Leu Val Val Val Asp Phe Met Met Thr Leu Leu Phe Met



This sequence shows 9 mutations when compared to the wild type *Bacillus circulans* xylanase sequence, Y53F, D83V, S84V, Y105F, R132M, R136M, A142L, I144L, and H149F (see Figure 7B). This DEE ground state shows 95% identity with the complete wild type *Bacillus circulans* xylanase sequence and has 40% identity in the designed positions with the wild type sequence.

5 Using the Monte Carlo technique a list of low energy sequences was generated. The analysis of the lowest 1000 protein sequences generated by Monte Carlo leads to the mutation pattern shown in Figure 7A. Out of the lowest 1000 sequences none has more than 12 mutations from the wild type sequence and out of the lowest 101 sequences none has more than 12 mutations. Thus, any protein sequence showing mutations at the positions according to Figure 7A will potentially generate a more  
10 stable and/or active XA protein. In particular those protein sequences found among the list of the lowest 101 MC generated sequences (data not shown) have a high potential to result in a more stable and/or active XA protein. A preferred XA sequence is shown in Figure 7B. A DNA library can be generated to mirror the probability table of Figure 7A that comprises at least one sequence that is more stable and/or active than wild type *Bacillus circulans* xylanase.

#### 15 PDA Calculations for the *Bacillus circulans* Xylanase Region around D83 (d)

A similar calculation as above was performed where T67, V82, W85, were excluded and D83 was considered as a CORE residue, but was still allowed to change its identity to all rotamers of the boundary group (see Figure 3).

20 The PDA calculation resulted in the following ground state sequence, which is identical to the one obtained above in (c):

53 66 68 81 83 84 101 105 132 136 138 142 144 149 169  
Phe Leu Leu Val Val Val Asp Phe Met Met Thr Leu Leu Phe Met

25 Using the Monte Carlo technique a list of low energy sequences was generated. The analysis of the lowest 1000 protein sequences generated by Monte Carlo leads to the mutation pattern shown in Figure 8A. This pattern is slightly different from the one shown in Figure 7A. Out of the lowest 1000 sequences none has more than 12 mutations from the wild type sequence and out of the lowest 101 sequences none has more than 12 mutations. Thus, any protein sequence showing mutations at the positions according to Figure 8A will potentially generate a more stable and/or active XA protein. In particular those protein sequences found among the list of the lowest 101 MC generated sequences

(data not shown) have a high potential to result in a more stable and/or active XA protein. A preferred XA sequence is shown in Figure 8B. A DNA library can be generated to mirror the probability table of Figure 8A that comprises at least one sequence that is more stable and/or active than wild type *Bacillus circulans* xylanase.

#### 5                    PDA Calculations for the *Bacillus circulans* Xylanase Region around D83 (e)

A similar calculation as above was performed where the boundary rotamer group without methionine was used for R132 AND R136 and D83 was considered as a BOUNDARY residue (see Figure 3).

The PDA calculation resulted in the following ground state sequence:

10                    53   66   67   68   81   82   83   84   85 101 105 132 136 138 142 144 149 169  
Phe Leu Thr Leu Val Val Val Val Phe Asp Phe Ala Leu Thr Leu Leu Phe Met

This sequence shows 10 mutations when compared to the wild type *Bacillus circulans* xylanase sequence, Y53F, D83V, S84V, W85F, Y105F, R132A, R136L, A142L, I144L, and H149F (see Figure 9B). This DEE ground state shows 95% identity with the complete wild type *Bacillus circulans* xylanase sequence and has 40% identity in the designed positions with the wild type sequence.

15                    Using the Monte Carlo technique a list of low energy sequences was generated. The analysis of the lowest 1000 protein sequences generated by Monte Carlo leads to the mutation pattern shown in Figure 9A. Out of the lowest 1000 sequences none has more than 13 mutations from the wild type sequence and out of the lowest 101 sequences none has more than 13 mutations. Thus, any protein sequence showing mutations at the positions according to Figure 9A will potentially generate a more  
20                    stable and/or active XA protein. In particular those protein sequences found among the list of the lowest 101 MC generated sequences (data not shown) have a high potential to result in a more stable and/or active XA protein. A preferred XA sequence is shown in Figure 9B. A DNA library can be generated to mirror the probability table of Figure 9A that comprises at least one sequence that is more stable and/or active than wild type *Bacillus circulans* xylanase.

#### 25                    PDA Calculations for the *Bacillus circulans* Xylanase Region around D83 (f)

A similar calculation as above(e) was performed where the boundary rotamer group without methionine was used for R132 AND R136 and D83 was considered as a CORE residue, but was still allowed to change its identity to all rotamers of the boundary group (see Figure 3).

The PDA calculation resulted in the following ground state sequence:

5        53   66   67   68   81   82   83   84   85 101 105 132 136 138 142 144 149 169  
Phe Leu Thr Leu Val Val Val Ala Phe Asp Phe Ala Leu Thr Leu Leu Phe Met

10        This sequence shows 10 mutations when compared to the wild type *Bacillus circulans* xylanase sequence, Y53F, D83V, S84A, W85F, Y105F, R132A, R136L, A142L, I144L, and H149F (see Figure 10B). This DEE ground state shows 95% identity with the complete wild type *Bacillus circulans* xylanase sequence and has 40% identity in the designed positions with the wild type sequence.

15        Using the Monte Carlo technique a list of low energy sequences was generated. The analysis of the lowest 1000 protein sequences generated by Monte Carlo leads to the mutation pattern shown in Figure 10A. Out of the lowest 1000 sequences none has more than 13 mutations from the wild type sequence and out of the lowest 101 sequences none has more than 13 mutations. Thus, any protein sequence showing mutations at the positions according to Figure 10A will potentially generate a more stable and/or active XA protein. In particular those protein sequences found among the list of the lowest 101 MC generated sequences (data not shown) have a high potential to result in a more stable and/or active XA protein. A preferred XA sequence is shown in Figure 10B. A DNA library can be generated to mirror the probability table of Figure 10A that comprises at least one sequence that is  
20        more stable and/or active than wild type *Bacillus circulans* xylanase.

Both (c) and (d) or (e) and (f) show that the ground state sequence is nearly independent of D83 being a CORE or BOUNDARY residue.

#### EXAMPLE 4

##### PDA Calculations for the *Bacillus circulans* Xylanase Helix Region (a)

25        Another region offering potential for higher thermostability and alkaline activity is the region around the helix (residue T147 to K154) in the *Bacillus circulans* xylanase. The following residues were included in the PDA calculation: G70, T72, I77, Y79, V81, K95, G96, V98, S100, D101, G102, G103, Y105, I107, T109, Y128, S130, R132, I144, F146, T147, N148, H149, V150, A152, W153, H156, G157, M158, L160, G161, W164, and Q167 (see Figure 2C). Residues G70, T72, I77, Y79, V81, S100,

Y105, I107, Y128, S130, I144, F146, H149, V150, and W153 were treated as CORE positions and residues K95, G96, V98, D101, G102, G103, T109, R132, T147, N148, A152, H156, G157, M158, L160, G161, W164, and Q167 as BOUNDARY positions. The rest of the protein was treated as a fixed template.

5 Rotamer groups that include all phobic amino acids except methionine (Ala, Val, Leu, Ile, Phe, Tyr, Trp) plus the wild type residue were assigned to the CORE positions. H149 was allowed to change to Ala, Val, Leu, Ile, Phe, Tyr, Trp, His, and Ser. The rotamer group containing boundary residues (Ala, Val, Ile, Ile, Phe, Tyr, Trp, Asp, Asn, Glu, Gln, Lys, Ser, Thr, His, Arg, Met) was assigned to positions R132 and M158; and the rotamer group containing all boundary residues except methionine was  
10 assigned to positions K95, V98, D101, T109, T147, N148, A152, H156, L160, W164, and Q167; the rotamer group containing all boundary residues except methionine but plus glycine was used for the glycine boundary residues.

In the PDA calculation an energy cutoff of 20 kcal/mol was used for the rotamer/template energy to exclude unfavorable rotamers. The van der Waals radius was scaled by a factor of 0.9 and the  
15 solvation model 2 as defined by Street and Mayo (supra) was used. The DEE method was employed to extract the ground state rotamer sequence from all the possible rotamer sequences. In those cases where DEE could not extract a single sequence, a Monte Carlo (MC) search was performed starting from the point where DEE stopped.

Thus, the following positions were included in the PDA design (see also Figure 3):

20 70 72 77 79 81 95 96 98 100 101 102 103 105 107 109 128 130 132 144 146  
Gly Thr Ile Tyr Val Lys Gly Val Ser Asp Gly Gly Tyr Ile Thr Tyr Ser Arg Ile Phe  
147 148 149 150 152 153 156 157 158 160 161 164 167  
Thr Asn His Val Ala Trp His Gly Met Leu Gly Trp Gln

The PDA calculation resulted in the following ground state sequence:

25 70 72 77 79 81 95 96 98 100 101 102 103 105 107 109 128 130 132 144 146  
Gly Thr Ile Phe Val Lys Ser Thr Val Asp Asp Ile Phe Ile Ile Val Ala Ala Leu Phe  
147 148 149 150 152 153 156 157 158 160 161 164 167  
Ile Glu Phe Ile Ser Trp Tyr Gly Ile Phe Gly Trp Glu

This sequence shows 21 mutations when compared to the wild type *Bacillus circulans* xylanase sequence, Y79F, G96S, V98T, S100V, G102D, G103I, Y105F, T109I, Y128V, S130A, R132A, I144L, T147I, N148E, H149F, V150I, A152S, H156Y, M158I, L160F, and Q167E (see Figure 11B). This DEE ground state shows 89% identity with the complete wild type *Bacillus circulans* xylanase sequence and has 36% identity in the designed positions with the wild type sequence.

Using the Monte Carlo technique a list of low energy sequences was generated. The analysis of the lowest 1000 protein sequences generated by Monte Carlo leads to the mutation pattern shown in Figure 11A. Out of the lowest 1000 sequences none has more than 23 mutations from the wild type sequence and out of the lowest 101 sequences none has more than 22 mutations. Thus, any protein sequence showing mutations at the positions according to Figure 11A will potentially generate a more stable and/or active XA protein. In particular those protein sequences found among the list of the lowest 101 MC generated sequences (data not shown) have a high potential to result in a more stable and/or active XA protein. A preferred XA sequence is shown in Figure 11B. A DNA library can be generated to mirror the probability table of Figure 11A that comprises at least one sequence that is more stable and/or active than wild type *Bacillus circulans* xylanase.

#### PDA Calculations for the *Bacillus circulans* Xylanase Helix Region (b)

A similar calculation as above (a) was performed for the helix region where the residues G70, G96, G103, Y105, S130, R132, H149, W153, and G157, which are conserved over family 11 xylanases, are assigned to the rotamer group WILD TYPE and kept in the CORE and BOUNDARY region.

The PDA calculation resulted in the following ground state sequence:

```

70  72  77  79  81  95  96  98 100 101 102 103 105 107 109 128 130 132 144 146
Gly Thr Ile Phe Val Lys Gly Val Val Asp Gly Gly Tyr Ile Ile Val Ser Arg Ile Phe

147 148 149 150 152 153 156 157 158 160 161 164 167
Ile Glu His Ile Ser Trp Tyr Gly Ile Phe Gly Trp Glu

```

This sequence shows 12 mutations when compared to the wild type *Bacillus circulans* xylanase sequence, Y79F, S100V, T109I, Y128V, T147I, N148E, V150I, A152S, H156Y, M158I, L160F, and Q167E (see Figure 12B). This DEE ground state shows 94% identity with the complete wild type

*Bacillus circulans* xylanase sequence and has 50% identity in the designed positions with the wild type sequence.

Using the Monte Carlo technique a list of low energy sequences was generated. The analysis of the lowest 1000 protein sequences generated by Monte Carlo leads to the mutation pattern shown in Figure 12A. Out of the lowest 1000 sequences none has more than 15 mutations from the wild type sequence and out of the lowest 101 sequences none has more than 13 mutations. Thus, any protein sequence showing mutations at the positions according to Figure 12A will potentially generate a more stable and/or active XA protein. In particular those protein sequences found among the list of the lowest 101 MC generated sequences (data not shown) have a high potential to result in a more stable and/or active XA protein. A preferred XA sequence is shown in Figure 12B. A DNA library can be generated to mirror the probability table of Figure 12A that comprises at least one sequence that is more stable and/or active than wild type *Bacillus circulans* xylanase.

#### PDA Calculations for the *Bacillus circulans* Xylanase Helix Region (c)

A similar calculation as above (a) residue F160 was not allowed to become any aromatic residue (Phe, Trp, Tyr, His) nor methionine and the two glycine at positions 102 and 103 were excluded from the design.

Thus, the following positions were included in the PDA design (see also Figure 3):

70 72 77 79 81 95 96 98 100 101 105 107 109 128 130 132 144 146  
Gly Thr Ile Tyr Val Lys Gly Val Ser Asp Tyr Ile Thr Tyr Ser Arg Ile Phe

147 148 149 150 152 153 156 157 158 160 161 164 167  
Thr Asn His Val Ala Trp His Gly Met Leu Gly Trp Gln

The PDA calculation resulted in the following ground state sequence:

70 72 77 79 81 95 96 98 100 101 105 107 109 128 130 132 144 146  
Gly Thr Ile Phe Val Lys Ser Thr Val Asp Phe Ile Ile Val Ala Met Leu Phe

147 148 149 150 152 153 156 157 158 160 161 164 167  
Ile Glu Phe Val Ser Trp Tyr Gly Ile Leu Gly Trp Glu

This sequence shows 17 mutations when compared to the wild type *Bacillus circulans* xylanase sequence, Y79F, G96S, V98T, S100V, Y105F, T109I, Y128V, S130A, R132M, I144L, T147I, N148E, H149F, A152S, H156Y, M158I, and Q167E (see Figure 13B). This DEE ground state shows 91% identity with the complete wild type *Bacillus circulans* xylanase sequence and has 45% identity in the designed positions with the wild type sequence.

Using the Monte Carlo technique a list of low energy sequences was generated. The analysis of the lowest 1000 protein sequences generated by Monte Carlo leads to the mutation pattern shown in Figure 13A. Out of the lowest 1000 sequences none has more than 20 mutations from the wild type sequence and out of the lowest 101 sequences none has more than 19 mutations. Thus, any protein sequence showing mutations at the positions according to Figure 13A will potentially generate a more stable and/or active XA protein. In particular those protein sequences found among the list of the lowest 101 MC generated sequences (data not shown) have a high potential to result in a more stable and/or active XA protein. A preferred XA sequence is shown in Figure 13B. A DNA library can be generated to mirror the probability table of Figure 13A that comprises at least one sequence that is more stable and/or active than wild type *Bacillus circulans* xylanase.

#### EXAMPLE 5

##### PDA Calculations for the *Bacillus circulans* Xylanase Active Site Region (a)

To demonstrate that computational pre-screening is feasible and will lead to a significant reduction in the number of sequences that have to be experimentally screened, initial calculations for the *B. circulans* xylanase with and without the substrate were performed. The PDB structure 1XNB of *B. circulans* xylanase and 1BCX for the enzyme substrate complex were used. 27 residues inside the binding site were visually identified as belonging to the active site. These 27 residues are Y5, Q7, Y9, D11, N35, V37, G39, D63, Y65, T67, Y69, W71, E78, Y80, V82, Y88, T110, R112, A115, I118, F125, Q127, W129, Y166, V168, A170, E172. 8 of these residues were regarded as absolutely essential for the enzymatic activity. These eight residues are Y9, N35, Y69, E78, R112, Q127, Y166, and E172. These positions were treated as wild type residues, which means that their conformation was allowed to change but not their amino acid identity.

Three of the 20 naturally occurring amino acids were not considered (cysteine, proline, and glycine). Therefore, 17 different amino acids were still possible at the remaining 19 positions; the problem yields  $17^{19} = 2.4 \times 10^{23}$  different amino acid sequences. This number is 10 orders of magnitude larger than what can be handled by state of the art directed evolution methods. Clearly these approaches cannot be used

to screen the complete dimensionality of the problem and consider all sequences with multiple substitutions. Therefore PDA calculations were performed to reduce the search space.

Thus, the following positions were included in the PDA design (see also Figure 3):

5      5    7    11   37   39   63   65   67   71   80   82   88 110 115 118 125 129 168 170  
Tyr Gln Asp Val Gly Asp Tyr Thr Trp Tyr Val Tyr Thr Ala Ile Phe Trp Val Ala

The PDA calculation resulted in the following ground state sequence:

5      5    7    11   37   39   63   65   67   71   80   82   88 110 115 118 125 129 168 170  
Tyr Glu Ile Asp Ala Trp Glu Glu Trp Leu Val Asn Asp Tyr Glu Met Ser Asp Ala

10      This sequence shows 15 mutations when compared to the wild type *Bacillus circulans* xylanase sequence, Y5W, Q7E, D11I, V37D, G39A, N63W, Y65E, T67E, Y80L, T110D, A115Y, I118E, F125M, W129S, and V168D (see Figure 14B).

15      Using the Monte Carlo technique a list of low energy sequences was generated. The analysis of the lowest 10,000 protein sequences generated by Monte Carlo leads to the mutation pattern shown in Figure 14A. Thus, any protein sequence showing mutations at the positions according to Figure 14A will potentially generate a more stable and/or active XA protein. In particular those protein sequences found among the list of the lowest 101 MC generated sequences (data not shown) have a high potential to result in a more stable and/or active XA protein. A preferred XA sequence is shown in Figure 14B. A DNA library can be generated to mirror the probability table of Figure 14A that comprises at least one sequence that is more stable and/or active than wild type *Bacillus circulans* xylanase.

## 20      Secondary Library Generation of a Xylanase

25      If we consider all the amino acids obtained from the PDA calculation (see above), including those with probabilities less than 1%, we obtain  $4.1 \times 10^{15}$  different amino acid sequences. This is a reduction by 7 orders of magnitude. If one only considers those amino acids that have at least a probability of more than 1% as shown in Figure 14A (1% criterion), the problem is decreased to  $3.3 \times 10^9$  sequences. If one neglects all amino acids with a probability of less than 5% (5% criterion) there are only  $4.0 \times 10^6$  sequences left. This is a number that can be easily handled by screening and gene shuffling techniques. Increasing the list of low energy sequences to 100,000 does not change these numbers significantly and the effect on the amino acids obtained at each position is negligible.



PDA Calculations for the *Bacillus circulans* xylanase with Active Site Region with Substrate (b)

For the PDA design with the substrate the PDB structure 1BCX was used (Wakarchuk et al., supra). In the PDA design the mutated position E172C of the PDB structure was forced back to the wild type glutamate.

The PDA calculation resulted in the following ground state sequence:

5        5    7    11   37   39   63   65   67   71   80   82   88   110   115   118   125   129   168   170  
Tyr Gln Asp Asp Ser Trp Glu Glu Trp Met Val Trp Asp Asp Ile Phe Leu Asp Thr

This sequence shows 12 mutations when compared to the wild type *Bacillus circulans* xylanase sequence, V37D, G39S, N63W, Y65E, T67E, Y80M, Y88W, T110D, A115D, W129L, V168D, and A170T (see Figure 15B).

10        Using the Monte Carlo technique a list of low energy sequences was generated. The analysis of the lowest 10,000 protein sequences generated by Monte Carlo leads to the mutation pattern shown in Figure 15a. Thus, any protein sequence showing mutations at the positions according to Figure 15A will potentially generate a more stable and/or active XA protein. In particular those protein sequences found among the list of the lowest 101 MC generated sequences (data not shown) have a high potential to result in a more  
15        stable and/or active XA protein. A preferred XA sequence is shown in Figure 15B. A DNA library can be generated to mirror the probability table of Figure 15A that comprises at least one sequence that is more stable and/or active than wild type *Bacillus circulans* xylanase.

This PDA calculation further reduced the number of amino acids found at each position. If we consider those amino acids with a probability higher than 5%, we obtain  $2.4 \times 10^6$  sequences (see Figure 15A).

20        These preliminary calculations show that PDA can significantly reduce the dimensionality of the problem and can bring it into the scope of gene shuffling and screening techniques.

## CLAIMS

We claim:

1. A non-naturally occurring XA protein comprising an amino acid sequence that is less than about 97% identical to a naturally occurring *Bacillus circulans* xylanase, wherein said XA protein has been modified to exhibit enhanced thermophilicity, alkalophilicity, or thermostability relative to said naturally occurring *Bacillus circulans* xylanase.
2. A non-naturally occurring XA protein comprising an amino acid sequence that has at least about 5 amino acid substitutions as compared to a *Bacillus circulans* xylanase sequence, wherein said XA protein has been modified to exhibit enhanced thermophilicity, alkalophilicity, or thermostability relative to said naturally occurring *Bacillus circulans* xylanase.
3. A non-naturally occurring XA conformer having a three dimensional backbone structure that substantially corresponds to the three dimensional backbone structure of *Bacillus circulans* xylanase, wherein the amino acid sequence of said conformer and said amino acid sequence of said *Bacillus circulans* xylanase are less than about 97% identical.
4. The conformer according to claim 3, wherein at least about 90% of the non-identical amino acids are in a core region of said conformer.
5. The conformer according to claim 4, wherein 100% of said non-identical amino acids are in a core region of said conformer.
6. A non-naturally occurring XA protein comprising at least three amino acid substitutions as compared to *Bacillus circulans* xylanase, wherein at least three of said substitutions are selected from the amino acid residues at positions selected from positions 7, 26, 28, 30, 39, 53, 58, 63, 64, 65, 67, 79, 80, 83, 84, 85, 88, 96, 98, 100, 102, 103, 105, 109, 110, 118, 128, 129, 130, 132, 136, 142, 144, 147, 148, 149, 150, 152, 156, 158, 160, 167, 168, 171, 176, 180, and 182.

7. The non-naturally occurring XA protein according to claim 6, wherein said XA protein has at least 5 amino acid substitutions.

8. The non-naturally occurring XA protein according to claim 6, wherein 3 of said at least three amino acid substitutions are at amino acid residues at positions 26, 28, 30, 53, 58, 64, 79, 105, 142, 171, 176, 180, and 182.

9. The non-naturally occurring XA protein according to claim 8, wherein said substitutions are selected from the group of substitutions consisting of Y26F, V28I, W30F, Y53F, W58F, G64V, Y79F, Y105F, A142L, T171L, S176A, S180A, and V182I.

10. The non-naturally occurring XA protein according to claim 6, wherein 3 of said at least three amino acid substitutions are at amino acid residues at position 53, 83, 84, 85, 105, 132, 136, 142, 144, and 149.

11. The non-naturally occurring XA protein according to claim 10, wherein said substitutions are selected from the group of substitutions consisting of Y53F, D83V, S84V, S84A, W85F, Y105F, R132M, R136M, R132A, R136L, A142L, I144L, and H149.

12. The non-naturally occurring XA protein according to claim 6, wherein 3 of said at least three amino acid substitutions are at amino acid residues at position 79, 96, 98, 100, 102, 103, 105, 109, 128, 130, 132, 144, 147, 148, 149, 150, 152, 156, 158, 160, and 167.

13. The non-naturally occurring XA protein according to claim 12, wherein said substitutions are selected from the group of substitutions consisting of Y79F, G96S, V98T, S100V, G102D, G103I, Y105F, T109I, Y128V, S130A, R132A, R132M, I144L, T147I, N148E, H149F, V150I, A152S, H156Y, M158I, L160F, and Q167E.

14. The non-naturally occurring XA protein according to claim 6, wherein 3 of said at least three amino acid substitutions are at amino acid residues at position 5, 7, 11, 37, 39, 63, 65, 67, 80, 81, 110, 115, 118, 125, 129, and 168.

15. The non-naturally occurring XA protein according to claim 13, wherein said substitutions are selected from the group of substitutions consisting of Y5W, Q7E, D11I, V37D, G39A, G39S, N63W, Y65E, T67E, Y80L, Y80M, T110D, A115Y, I118E, F125M, W129E, W129L, W129S, V168D, and A170T.

16. A recombinant nucleic acid encoding the non-naturally occurring XA protein of claim 1, 2 or 6.

5 17. An expression vector comprising the recombinant nucleic acid of claim 16.

18. A host cell comprising the recombinant nucleic acid of claim 16.

19. A host cell comprising the expression vector of claim 17.

20. A method of producing a non-naturally occurring XA protein comprising culturing the host cell of claim 18 under conditions suitable for expression of said nucleic acid.

10 21. The method according to claim 20 further comprising recovering said XA protein.

22. A bleaching agent comprising as an active ingredient an XA protein..

23. A method for bleaching pulp, said method comprising the step of contacting pulp to be bleached with the bleaching agent of claim 22.

15 24. The method of claim 23, further comprising the step of chemical bleaching and/or an alkali extraction before, after or during said step of contacting pulp with said bleaching agent.

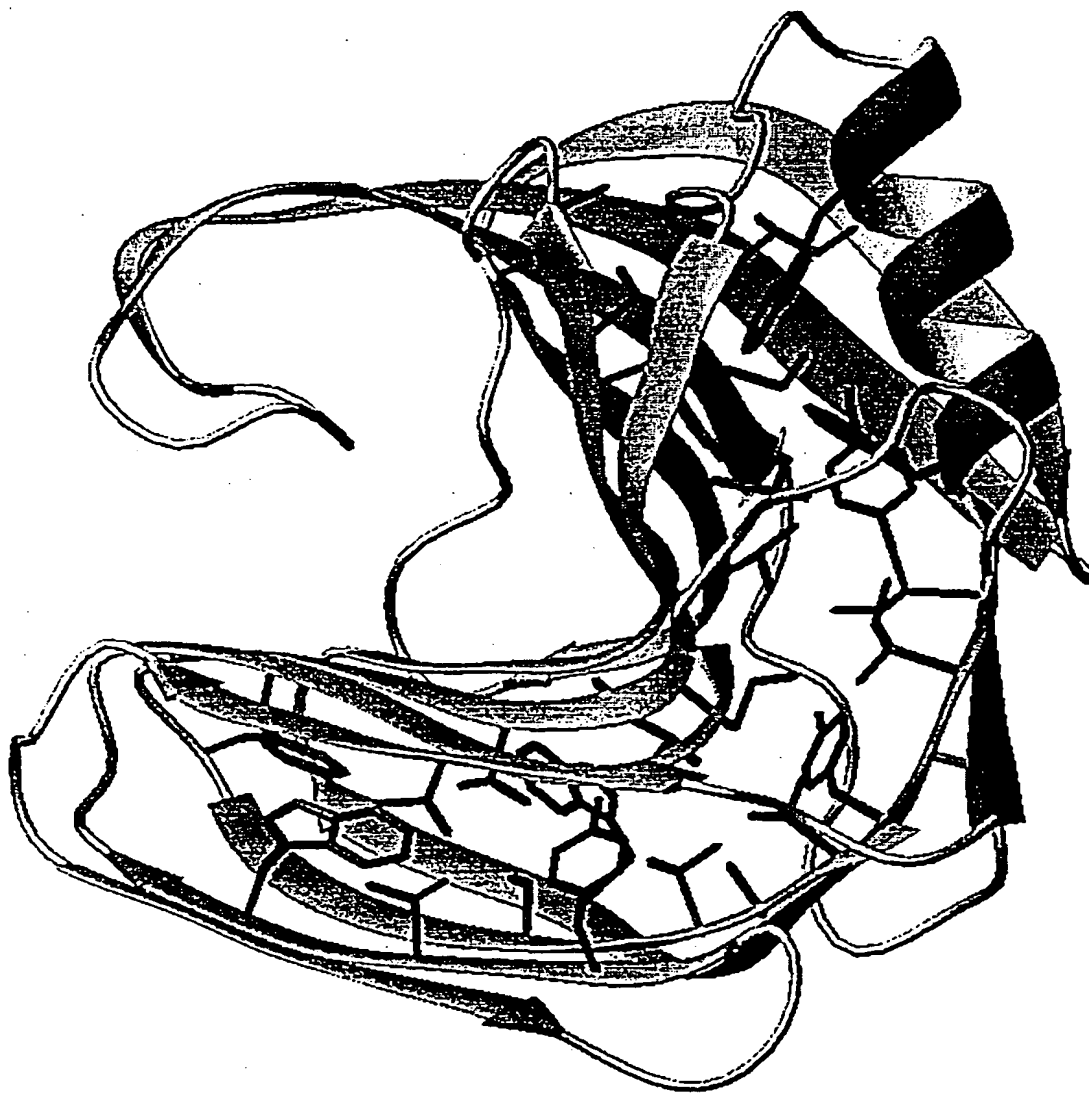
**A** 1 MFKFKKNFLV GLSAALMSIS LFSATASAAS TDYWQNWTDG GGIVNAVNGS  
 51 GGNYSVNWSN TGNFVVGKW TTGSPFRTIN YNAGVWAPNG NGYLTLYGWT  
 101 RSPLIEYYV DSWGTYRPTG TYKGTVKSDG GTYDIYTTTR YNAPSIDGDR  
 151 TTFTQYWSVR QSKRPTGSNA TITFTNHVNA WKSHGMNLGS NWAYQVMATE  
 201 GYQSSGSSNV TVW

**B** 1 ASTDYQNWNT DGGGIVNAVN GSGGNYSVNW SNTGNFVVGK GWTGSPFRT  
 EEEEE S S EEEEE STTEEEEE ES S EEEEE EESS TT E  
 51 INYNAGVWAP NGNGYLTLYG WTRSPLIEYY VVDSWGTYRP TGTYKGTVKS  
 EEEEEEEEE ES EEEEEEE EEETTTEEE EE EESS EE EEEEE  
 101 DGGTYDIYTT TRYNAPSIDG DRTTFTQYWS VRQSKRPTGS NATITFTNHV  
 TTEEEEEEE EESSEE TTS SEE EEEEE EESS SSS EEEEEHHHHH  
 151 NAWKSHGMNL GSNWAYQVMA TEGYQSSGSS NVTWV  
 HHHHHTT SEE EEEE EEEES EEEE EEEE

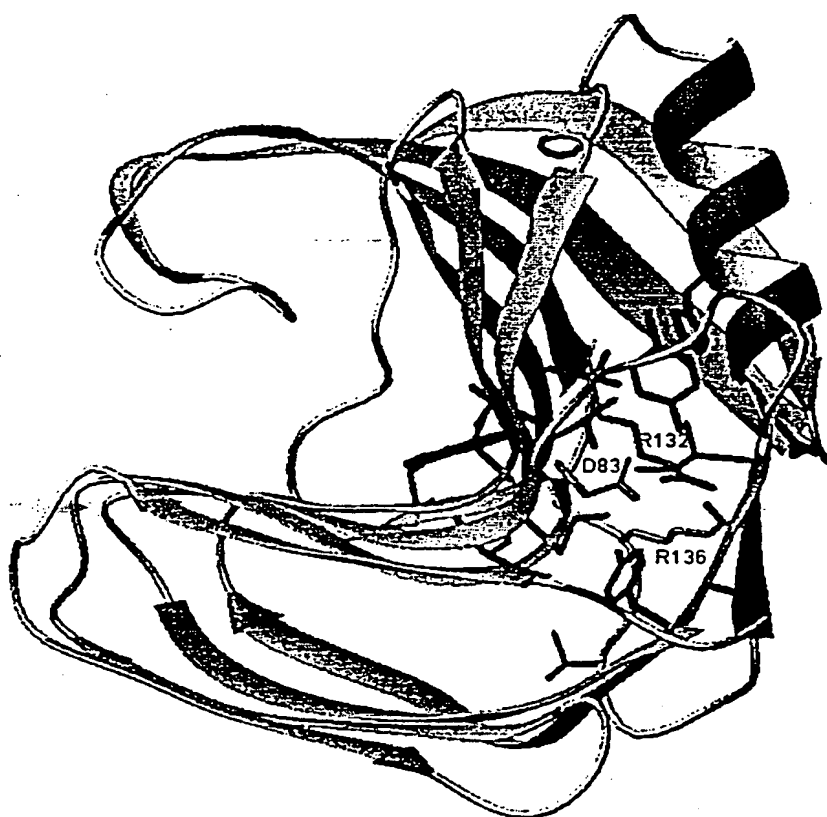
**C**

1 gaattcgggtg atgatggatt cgatgcgctt tagttctgag gtgatcacgt tgaagtaaag  
 61 ggcgtagtct cctcgcacac tccctttcag aagctgaatg aagcctttaa gagcagtcac  
 121 cggatttctg atttcatgag caataccggc tgcgagttcg ccgacaacat gaagggtatc  
 181 tgatttcgca ggcgctcctc caattccttt cgctctgtta cgtctttaaa gatagccaag  
 241 ttcataatatt gaataatatt ccgtttaaat gagaattcgt ggtattatac tgaaggggac  
 301 gatcaaaagc tttggcggtta gtaattaaaa atgttttaaa tgtatacgag tgctgcctca  
 361 aagttggaaa aaatattata ggaggtaaca tatgtttaag tttaaaaaga atttcttagt  
 421 tggattatcg gcagctttaa tgagtattag cttgttttcg gcaaccgcct ctgcagctag  
 481 cacagactac tggcaaaatt ggactgatgg ggcgggtata gtaaaccgtg tcaatgggtc  
 541 tggcggaat tacagtgtta attggtctaa taccggaaat tttgttgttg gtaaagggtg  
 601 gactacaggt tcgccattta ggacgataaa ctataatgcc ggagtttggg cgccgaatgg  
 661 caatggatat ttaactttat atggttggaac gagatcacct ctcatagaat attatgtagt  
 721 ggattcatgg ggtacttata gacctactgg aacgtataaa ggtactgtaa aaagtgatgg  
 781 gggtacatat gacatatata caactacacg ttataacgca ccttccattg atggcgatcg  
 841 cactactttt acgcagtact ggagtgttcg ccagtcgaag agaccaactg gaagcaacgc  
 901 tacaatcact ttcacgaatc atgtgaacgc atggaagagc catggaatga atctgggcag  
 961 taattgggct taccaagtca tggcgacaga aggatatcaa agtagtgga gttctaactg  
 1021 aacagtgtgg taacagatca tccttaatca ggggtagcta acgggctgct gatcggttct  
 1081 tgagaagttt tataatcaat gattattaaa atcgtagta agggttaaag gttgttttct  
 1141 actaggtgaa cggccttgca attgctggag gtaggggtatt ctccatctgg ttttataact  
 1201 tttcctatag gttaatagaa tggattttaa atgagaatgc tacaattttt tctagtcagc  
 1261 gcttgctcac aacagacacc tttacataac tcttctttat caaacataag ccttattcaa  
 1321 aataaaaata tctagtagtt gacctgcag

**Figure 1**



**FIGURE 2A**



**FIGURE 2B**

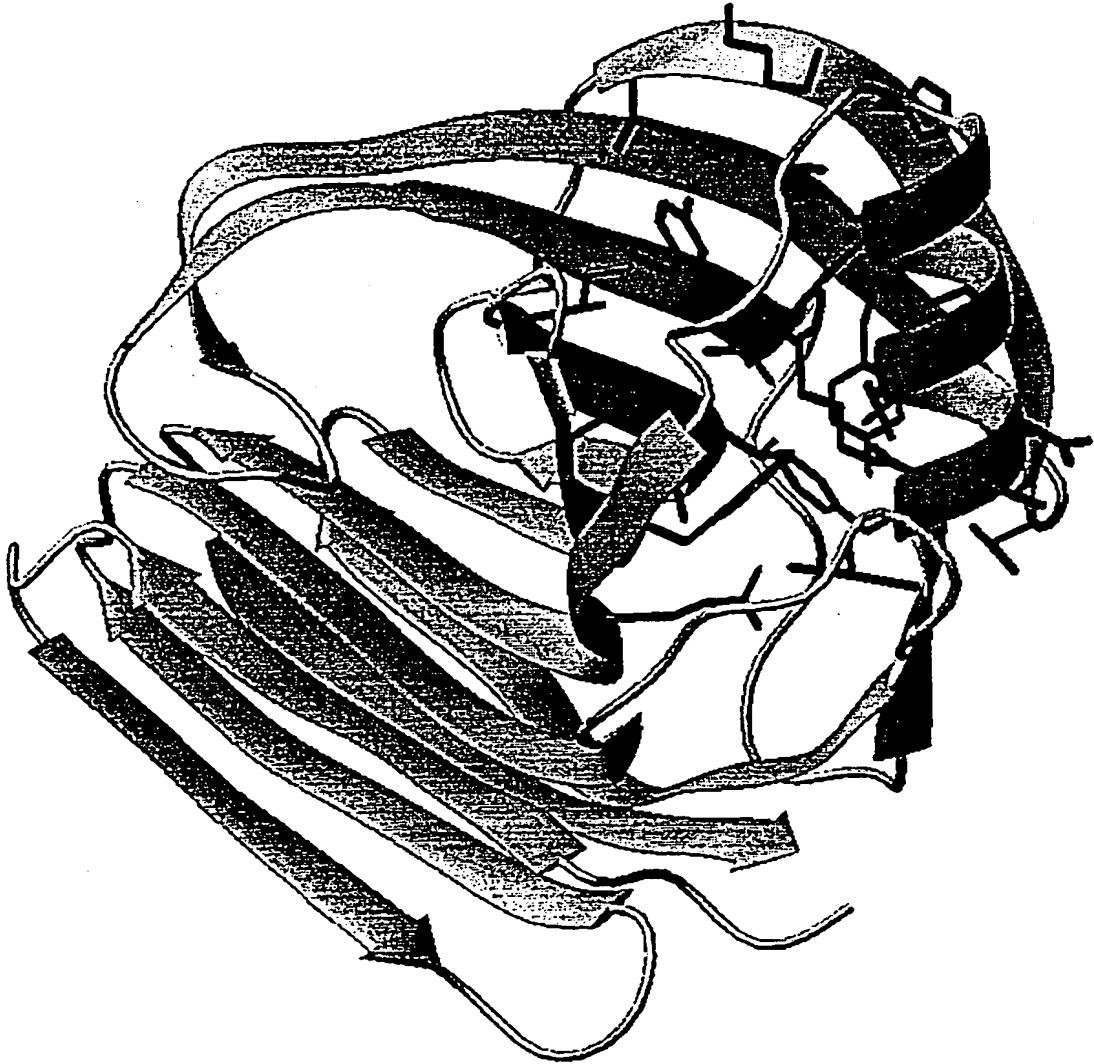
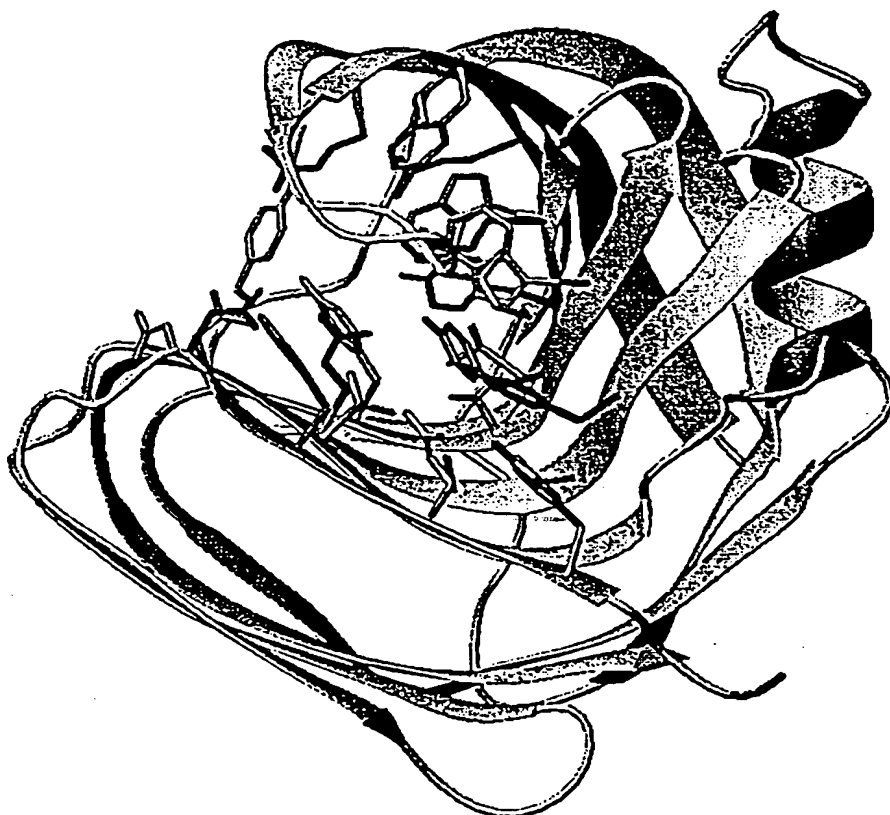


FIGURE 2C





**FIGURE 2D**

*Bacillus circulans* xylanase CORE

26 28 30 36 38 51 53 55 58 62 64 66 68 70 72 77 79 81 105 107  
 Tyr Val Trp Phe Val Ile Tyr Ala Trp Gly Gly Leu Leu Gly Thr Ile Tyr Val Tyr Ile

130 142 144 146 153 169 171 173 176 178 180 182 184  
 Ser Ala Ile Phe Trp Met Thr Gly Ser Gly Ser Val Val

*Bacillus circulans* xylanase Region around D83 (a, b, e, f)

53 66 67 68 81 82 83 84 85 101 105 132 136 138 142 144 149 169  
 Tyr Leu Thr Leu Val Val Asp Ser Trp Asp Tyr Arg Arg Thr Ala Ile His Met

*Bacillus circulans* xylanase Region around D83 (c, d)

53 66 68 81 83 84 101 105 132 136 138 142 144 149 169  
 Tyr Leu Leu Val Asp Ser Asp Tyr Arg Arg Thr Ala Ile His Met

*Bacillus circulans* xylanase Helix Region (a, b)

70 72 77 79 81 95 96 98 100 101 102 103 105 107 109 128 130 132 144 146  
 Gly Thr Ile Tyr Val Lys Gly Val Ser Asp Gly Gly Tyr Ile Thr Tyr Ser Arg Ile Phe

147 148 149 150 152 153 156 157 158 160 161 164 167  
 Thr Asn His Val Ala Trp His Gly Met Leu Gly Trp Gln

*Bacillus circulans* xylanase Helix Region (c)

70 72 77 79 81 95 96 98 100 101 105 107 109 128 130 132 144 146  
 Gly Thr Ile Tyr Val Lys Gly Val Ser Asp Tyr Ile Thr Tyr Ser Arg Ile Phe

147 148 149 150 152 153 156 157 158 160 161 164 167  
 Thr Asn His Val Ala Trp His Gly Met Leu Gly Trp Gln

*Bacillus circulans* xylanase Active Site Region (a, b)

5 7 11 37 39 63 65 67 71 80 82 88 110 115 118 125 129 168 170  
 Tyr Gln Asp Val Gly Asp Tyr Thr Trp Tyr Val Tyr Thr Ala Ile Phe Trp Val Ala

**FIGURE 3**

A

	26	28	30	36	38	51	53	55	58	62	64
>90%	F			F			F	A	F	G	V
>50%			F		V	I					
>30%		I,A			I						
>10%		V	W			L					
>1%		S		L		V	Y,W	S	A		A
<1%	Y	L,W	Y	Y					S		

	66	68	70	72	77	79	81	105	107	130	142
>90%	L	L	G	T			V		I	S	
>50%					I	F		F			L
>30%											A
>10%						W		Y			
>1%		V			V,L	Y	I		V	A	S
<1%									L		V

	144	146	153	169	171	173	176	178	180	182	184
>90%		F	W		L	G	A	G			V
>50%	I			M					A	I	
>30%											
>10%				L					F	V	
>1%	V,L	I,Y			I	A	S				
<1%		V	L		V				S	L	

B

1 ASTDYWQ~~N~~WT DGGGIVNAVN GSGGNFSINE SNTGNFVVGK GWTTGSPFRT  
 51 INFNAGVFAP NGNVYLTLYG WTRSPLIEFY VVDSWGTYRP TGTYKGTVKS  
 101 DGGTFEDIYTT TRYNAPSIDG DRTTFTQYWS VRQSKRPTGS NLTITFTNHV  
 151 NAWKSHGMNL GSNWAYQVMA LEGYQASGSA NITVW

FIGURE 4

**A**

	53	66	67	68	81	82	83	84	85	101	105
>90%	F			L	V	V	V				F
>50%		L						V	F	D	
>30%			T					A	Y		
>10%		F	A,D							N	
>1%			S,N	V	A	L	F	S,T,D			Y
<1%				I		T,D				A	

	132	136	138	142	144	149	169
>90%		M			L	F	M
>50%	M		T	L			
>30%							
>10%	L,A		V,D	I			
>1%		K	L	A,V	A,I	H	L
<1%		L	A			Y	

**B**

1 ASTDYQ~~N~~WT DGGGIVNAVN GSGGNYSVNW SNTGNFVVGK GWTTGSPFRT  
 51 INE~~N~~AGVWAP NGNGYLTLYG WTRSPLIEYY VVV~~V~~FTYRP TGTYKGTVKS  
 101 DGGT~~F~~DIYTT TRYNAPSIDG DRTTFTQYWS VM~~Q~~SK~~M~~PTGS NL~~T~~L~~T~~FTN~~F~~V  
 151 NAWKSHGMNL GSNWAYQVMA TEGYQSSGSS NVT~~V~~W

**FIGURE 5**

**A**

	53	66	67	68	81	82	83	84	85	101	105
>90%	F			L	V	V	V		F	D	F
>50%		L						A,V	Y		
>30%			T							N	
>10%		F	A,D					S,T,D			Y
>1%			S,N	V	A	L	T,D			A	
<1%						T,D					

	132	136	138	142	144	149	169
>90%		M			L	F	M
>50%	M		T	L			
>30%							
>10%	A,L		D,V	I			
>1%		L	A,L,S	V,A	I	H	L
<1%	S	K	N			Y	

**B**

1 ASTDYWQ~~N~~WT DGGGIVNAVN GSGGNYSVNW SNTGNFVVGK GWTTGSPFRT

51 INENAGVWAP NGNGYLTLYG WTRSPLIEYY VVVVEGTYRP TGT~~Y~~KGTVKS

101 DGGTEDIYTT TRYNAPSIDG DRTTFTQYWS VMQSKMPTGS NLTLTFTNEV

151 NAWKSHGMNL GSNWAYQVMA TEGYQSSGSS NVTVW

**FIGURE 6**

**A**

	53	66	68	81	83	84	101	105
>90%	F		L	V	V			
>50%		L				V,A	D	F
>30%							N	Y
>10%		F					A,S	
>1%			V,I	A	F	S,T,D		
<1%	W				M			

	132	136	138	142	144	149	169
>90%		M					M
>50%	M		T		L	F	
>30%				L,I			
>10%	L,A		V,D	A	I	H	
>1%	S	K,L	L,A	V	A	Y	L
<1%	D,E	E,F	S,N				

**B**

1 ASTDYWQ~~N~~WT DGGGIVNAVN GSGGNYSVNW SNTGNFVVGK GWTTGSPFRT  
 51 INENAGVWAP NGNGYLTLYG WTRSPLIEYY VVVWGTYRP TGT~~Y~~KGTVKS  
 101 DGGTFDIYTT TRYNAPSIDG DRTTFTQYWS VMQSKMPTGS NLTLTFTNEV  
 151 NAWKSHGMNL GSNWAYQVMA TEGYQSSGSS NVTVW

**FIGURE 7**

**A**

	53	66	68	81	83	84	101	105
>90%	F			V				
>50%		L	L		V		D	F
>30%					D	A,V		
>10%		F					N	Y
>1%			V,I	A	T	S,D,T	A	
<1%	W				A,E		S	

	132	136	138	142	144	149	169
>90%		M					M
>50%	M			L	L	F	
>30%			T	I			
>10%	L,A		D,V		I	H	
>1%	S	K,L	A,L,S N	V,A	V		L
<1%	D		H			Y	

**B**

1 ASTDYWQ~~N~~WT DGGGIVNAVN GSGGNYSVNW SNTGNFVVGK GWTTGSPFRT  
 51 IN~~F~~NAGVWAP NGNGYLTLYG WTRSPLIEYY VV~~V~~VWGTYRP TGTYKGTVKS  
 101 DGGT~~F~~DIYTT TRYNAPSIDG DRTTFTQYWS V~~M~~QSK~~M~~PTGS N~~L~~T~~L~~TFTN~~F~~V  
 151 NAWKSHGMNL GSNWAYQVMA TEGYQSSGSS NVTVW

**FIGURE 8**

A

	53	66	67	68	81	82	83	84	85	101	105
>90%	F	L		L	V	V	V				
>50%								V	F	D	F
>30%			T					A	Y		
>10%			A,D				F			N	Y
>1%	W	F	S,N	V	A	L,T		S,T,D		A	
<1%				I		D				S	

	132	136	138	142	144	149	169
>90%						F	M
>50%	A	L	T	L	L		
>30%	L						
>10%		K	V,D	I			
>1%	S	I,F,E,A	A,L	V,A,F	A,I	H,Y	L
<1%			S				

B

1 ASTDYWQNWT DGGGIVNAVN GSGGNYSVNW SNTGNFVVVK GWTGSPFRT  
 51 INFNAGVWAP NGNGYLTLYG WTRSPLIEYY VVVVEGTYRP TGTYKGTVKS  
 101 DGGTFEDIYTT TRYNAPSIDG DRTTFTQYWS VAQSKLPTGS NLTLTFTNEV  
 151 NAWKSHGMNL GSNWAYQVMA TEGYQSSGSS NVTWV

FIGURE 9



**A**

	53	66	67	68	81	82	83	84	85	101	105
>90%	F	L		L	V	V	V				F
>50%								A	F	D	
>30%			T					V	Y		
>10%			A,D							N	
>1%		F	S,N	V,I	A	L,T,D	D	S,T,D		A	Y
<1%	W						T			S	

	132	136	138	142	144	149	169
>90%					L	F	M
>50%	A	L		L			
>30%			T				
>10%	L	I	D,V	I			
>1%	S	K,F,E	A,S,N	V,A	I	H	L
<1%		A	L,Hsp	F		Y	

**B**

1 ASTDYWQNWT DGGGIVNAVN GSGGNYSVNW SNTGNFVVGK GWTTGSPFRT  
 51 INENAGVWAP NGNGYLTLYG WTRSPLIEYY VVVAFGTYRP TGTYKGTVKS  
 101 DGGTEDIYTT TRYNAPSIDG DRTTFTQYWS VAQSKLPTGS NLTLTFTNFV  
 151 NAWKSHGMNL GSNWAYQVMA TEGYQSSGSS NVTVW

**FIGURE 10**

**A**

	70	72	77	79	81	95	96	98	100	101	102
>90%	G	T	I	F	V		S			D	D
>50%						K		T	V		
>30%											
>10%						L,I		V,D,A	A		
>1%			L,V		I	E,Q,V	G				
<1%						R		E			

	103	105	107	109	128	130	132	144	146	147	148
>90%	I	F				A	A	L	F		E
>50%			I		V					I	
>30%				V	I					D	
>10%			L	I,K,E							
>1%				T,Q,L							
<1%				D,R	L			I			Q

	149	150	152	153	156	157	158	160	161	164	167
>90%	F	I		W		G		F	G	W	E
>50%			S				I				
>30%					Y						
>10%			Y,A		F,E		L,M				
>1%		V		F	V,I,L, Q,N		V	L			
<1%	H		W				E				

**B**

1 ASTDYWQ~~Q~~WT DGGGIVNAVN GSGGNYSVNW SNTGNEFVVGK GWTTGSPERT  
 51 INYNAGVWAP NGNGYLTLYG WTRSPLIEFY VDSWGTYP TGTYK~~STTKV~~  
 101 ~~DDIT~~EDIYIT TRYNAPSIDG DRTTFTQVWA VAQSKRPTGS NATLTFIEFI  
 151 NSWKS~~Y~~GINF GSNWAYEVMA TEGYQSSGSS NVTW

**FIGURE 11**

**A**

	70	72	77	79	81	95	96	98	100	101	102
>90%	G*	T	I	F	V	K	G*		V	D	G*
>50%								V			
>30%											
>10%								E			
>1%			V			L,E		T			
<1%						I		L			

	103	105	107	109	128	130	132	144	146	147	148
>90%	G	Y*				S*	R*		F		
>50%			I		V			I			E
>30%				I,V							
>10%			L	E	I			V		D,E,I	
>1%				K,T,Q						V,Q,L, A,K,N,S	Q,K,R, D,A
<1%				D							S,L

	149	150	152	153	156	157	158	160	161	164	167
>90%	H*	I		W*		G*		F	G	W	E
>50%			A				I				
>30%			S		Y						
>10%					L,Q						
>1%					F,N,E,K		M,L				
<1%		V	D		I		V	L			

**B**

1 ASTDYWQ~~N~~WT DGGGIVNAVN GSGGNYSVNW SNTGNFVVGK GWTTGSPFRT  
 51 INYNAGVWAP NGNGYLTLYG WTRSPLIEFY VVDSWGTYRP TGTYKGTVKY  
 101 DGGTYDIYIT TRYNA~~P~~SIDG DRTTFTQVWS VRQSKRPTGS NATITFIEHI  
 151 NSWKS~~Y~~G~~I~~N~~F~~ GSNWAYE~~V~~MA TEGYQSSGSS NVTW

**FIGURE 12**

**A**

	70	72	77	79	81	95	96	98	100	101
>90%	G	T	I	F			S			D
>50%					V	K			V	
>30%								T		
>10%					I			V,D	A	
>1%			L			I,L,E,Q	G	A,E		
<1%			V			V				

	105	107	109	128	130	132	144	146	147	148
>90%	F				A	M		F		E
>50%		I		V			L		I	
>30%			V,I						D	
>10%		L	E	I			I			
>1%			K,T,L,Q	L				Y		
<1%		V	R,D							

	149	150	152	153	156	157	158	160	161	164	167
>90%	F	V		W		G		L	G	W	E
>50%			S		Y		I				
>30%											
>10%			Y,A		E,F		L				
>1%	H	I		F	I,V,L,Q		M,V				
<1%			W		N						

**B**

1 ASTDYWQ~~N~~WT DGGGIVNAVN GSGGNYSVNW SNTGNEFVVGK GWTGSPFRT  
 51 INYNAGVWAP NGNGYLTLYG WTRSPLIEFY VVDSWGTYRP TGTYKSTTKV  
 101 DGGT~~F~~DIYIT TRYNAPSIDG DRTTFTQVWA VMQSKRPTGS NATLTFIEFV  
 151 NSWKS~~Y~~G~~I~~NL GSNWAYEVMA TEGYQSSGSS NVTWV

**FIGURE 13**

**A**

WT	PDA Probability Distribution							
5 Y	W 37.2%	F 25.8%	Y 22.9%	H 14.0%				
7 Q	E 69.1%	L 30.2%	V 10.1%	M 7.9%	L 6.4%	E 5.3%	T 4.2%	
11 D	I 41.2%	D 10.7%	Y 2.6%	F 2.1%	N 1.9%	S 1.9%	A 1.1%	
		Q 3.8%	V 21.4%	S 12.8%	I 4.1%	E 1.0%		
37 V	D 29.9%	M 29.4%						
39 G	A 99.8%							
63 N	W 91.2%	Q 6.7%	A 1.4%					
65 Y	E 91.7%	L 4.9%	M 3.4%					
67 T	E 81.0%	D 12.3%	L 3.9%	A 1.7%				
71 W	V 37.8%	F 25.5%	W 8.5%	M 6.0%	D 5.8%	E 4.3%	I 1.0%	
80 Y	M 32.4%	L 31.5%	F 19.0%	I 5.9%	Y 5.7%	E 3.7%		
82 V	V 88.6%	D 11.0%						
88 Y	N 91.1%	K 6.6%	W 1.3%					
110 T	D 99.9%							
115 A	A 35.6%	Y 27.8%	T 14.4%	D 10.2%	S 9.2%	F 2.6%		
118 I	E 92.2%	D 2.6%	I 2.0%	A 1.7%				
125 F	F 79.4%	Y 11.8%	M 7.3%	L 1.5%				
129 W	E 91.3%	S 8.6%						
168 V	D 98.1%							
170 A	A 78.7%	S 17.6%	D 3.7%					

**B**

1 ASTDWWENWT IGGGIVNAVN GSGGNYSVNW SNTGNFDVAK GWTTGSPFRT  
 51 INYNAGVWAP NGWGELELYG WTRSPLIEYL VVDSWGTNRP TGTYKGTVKS  
 101 DGTTYDIYTD TRYNYPSEDG DRTTMTQYSS VRQSKRPTGS NATITFTNHV  
 151 NAWKSHGMNL GSNWAYQDMA TEGYQSSGSS NVTVW

**FIGURE 14**

**A**

WT	PDA Probability Distribution							
5 Y	Y 69.2%	W 17.0%	H 7.3%	F 6.0%				
7 Q	Q 78.1%	E 18.0%	L 3.9%					
11 D	D 97.1%							
37 V	V 50.9%	D 33.9%	S 5.4%	A 1.2%	L 1.0%			
39 G	S 80.6%	A 19.4%						
63 N	W 92.2%	D 3.9%	Q 2.9%					
65 Y	E 91.1%	L 8.7%						
67 T	E 92.8%	L 5.2%						
71 W	W 62.6%	E 13.3%	M 11.0%	S 6.9%	D 4.0%			
80 Y	M 66.4%	F 13.6%	E 10.7%	I 6.0%	L 1.3%			
82 V	V 86.0%	D 12.8%						
88 Y	W 55.1%	Y 15.9%	N 11.4%	F 9.5%	K 1.9%	Q 1.4%	D 1.4%	M 1.4%
110 T	D 99.9%							
115 A	D 47.1%	S 27.8%	T 17.1%	A 7.9%				
118 I	I 47.6%	D 43.0%	E 3.6%	V 2.5%	A 1.4%			
125 F	Y 51.1%	F 43.3%	L 3.4%	M 2.0%				
129 W	L 63.2%	M 28.1%	E 7.5%					
168 V	D 98.2%							
170 A	T 92.3%	A 5.9%						

**B**

1 ASTDYWQ~~N~~WT DGGGIVNAVN GSGGNYSVNW SNTGNFDVSK GWTTGSPFRT  
 51 INYNAGVWAP NGWGELELYG WTRSPLIEYM VVDSWGTYRP TGTYKGTVKS  
 101 DGGTYDIYTD TRYNDPSIDG DRTTFTQYLS VRQSKRPTGS NATITFTNHV  
 151 NAWKSHGMNL GSNWAYQDMT TEGYQSSGSS NVTVW

**FIGURE 15**

**A**

MFKFKKNFVLGLSAAALMSISLFSATASAASTDYQONWTDGGGI VNAVNGSGGNYSVNWSNTGNFVVGKGW  
 TTGSPFRTINYNAGVWAPNGNGYLTLYGWTRSPLIEYYVVDSWGTYRPTGT YKGT VKSDGGTYDIYTTTR  
 YNAPSIDGDRTTFTQYWSVRQSKRPTGSNATITFTNHVNAWKSHGMNLGSNWAYQVMATEGYQSSGSSNV  
 TVW

**B**

mnlrklrllf vmcigltilil tavpaharti tnnemgnhsg ydyelwkdyg ntsmtlnngg  
 afsagwnnig nalfrkgkkf dstrthhqlg nisinynasf npggnsylcv ygwtsplae  
 yyivdswgty rptgaykgsf yadggtydiy ettrvnqpsi igiatfkqyw svrqtkrtsq  
 tvsvsahfrk weslgmpmgk myetaftveg yqssgsanvm tnqlfign

**C**

mnllvqprrr rrgpvtllvr sawavalaal aalmlpgtaq adtvvttnqe gtnngyyysf  
 wtdsqgtvsm nmgsqqyst swrntgnfva gkgwanggrr tvqysgsfnp sgnaylalyg  
 wtsnplveyy ivdnwgytrp tgeykgvtvs dggtydiykt trvnkpsveg trtfdqywsv  
 rqskrtggti ttgnhfdawa ragmplgnfs yymimategy qssgtssinv ggtgggdsgg  
 gdnggggggc tatvsagqkw gdrynlvsv sgasdwtvtn nvpapakvls nwnvnasyps  
 aqtltarlng sgnnwgatiq ananwtwpsv scsag

**D**

mqqdgtqqdr ikqspaplng msrrgflgga gtlalatasg lllpgtahaa ttittnqtgt  
 dgmyysfwtd gggsvsmtln gggysystqwt ncnfvgagkg wstgdgnvry ngyfnpvgng  
 ygclygwtsn plveyyivdn wgsyrptgty kgtvssdggt ydiyqttryn apsvegktf  
 qqywsvrqsk vtsgsgtitt gnhfdawara gmnmgqfryy mimategyqs sgssnitvsg

**E**

MLRRKVIFTVLATLVMTSLTIVDNTAFAATNLNTTESTFSKEVLSTQKTYSAFNTQAAPKTITSNEIGVN  
 GGYDYELWKDYGNTSMTLKNNGGAFSCQWSNIGNALFRKGKKFNDTQTYKQLGNISVNYDCNYQPYGNSYL  
 CVYGTSSPLVEYYIVDSWGSWRPPGGTSKGTITVDGGIYDIYETTRINQPSIQGNTTFKQYWSVRRTKR  
 TSGTISVSKHFAAWESKGMPLGKMHETAFNIEGYQSSGKADVNSMSINIGK

**FIGURE 16**

**F**

sgtpsstgtd ggyyyswtd gagdatyqnn gggstyltws gnngnlvggk gwnpgaasrs  
isysgtyqpn gnsylsvygw trsslieyyi vesygsydp saashkgsvt cngatydils  
twrynapsid gtqtfeqfws vrnpkkapgg sisgtvdivqc hfdawkglgm nlgsehnyqi  
vategyqssg tatitvt

**G**

qtigpgtgfn ngyfysywnd ghggvtytng pggqfsvnws nsgnfvggk wqpgtknkvi  
nfsgtynpng nsylsvygw rnplieyyiv enfgtynpst gatklgevts dgsvydiyrt  
qrvnqpsiig tstfyqywsv rrthrssgsv ntanhfnawa qqgltlgtmd yqivavegyf  
ssgsasitvs

**H**

qtigpgtgys ngyyysywnd ghagvtytng gggstfvnws nsgnfvagk wqpgtknkvi  
nfsgsynpng nsylsiygws rnplieyyiv enfgtynpst gatklgevts dgsvydiyrt  
qrvnqpsiig tatfyqywsv rrrhrssgsv ntanhfnawa shgltlgtmd yqivavegyf  
ssgsasitvs

**I**

asinydqnyq tggqvsysps ntgfsvnwnt qddfvvgvgw ttgssapinf ggsfsvnsgt  
gllsvygwst nplveyyime dnhnypaggt vkgtvtsdga tytiwentrv nepsiqgtat  
fnqyisvrns prtsgtvtvq nhfnawaslg lhlqgmnyqv vavegwggsg sasqsvsn

**J**

xtiqpgtgyn ngyfysywnd ghggvtytng pggqfsvnws nsgnfvggk wqpgtknkvi  
nfsgsynpng nsylsvygw rnplieyyiv enfgtynpst gatklgevts dgsvydiyrt  
qrvnqpsiig tatfyqywsv rrrhrssgsv ntanhfnawa qqgltlgtmd yqivavegyf  
ssgsasitvs

**FIGURE 16**



**K**

gttptnsegwh dgyyyswwsd gggdstytnn sggtyeitwg nggnlvggkg wnpglnarai  
hftgvyqpng tsylsvygtw rnplveyyiv enfgssnpss gstdlgtvsc dgstytlgqs  
trynapsidg tqtfngqysv rqdkrsgtv qtgchfdawa saglnvtgdh yyqivategy  
fssgyariv advg

**L**

xttpnsegwh dgyyyswwsd ggaqatytnl eggtyeiswg dggnlvggkg wnpglnarai  
hfegvyqpng nsylavygwt rnplveyyiv enfgtydpss gatdlgtvec dgsiyrilgt  
trvnapsidg tqtfngqysv rqdkrsgtv qtgchfdawa raglnvngdh yyqivategy  
fssgyariv advg

**M**

saginyvqny ngnlgdftyd esagtfsmyw edgvssdfvv glgwttgssn aitysaeysa  
sgsasyldvy gwnypqaey yivedygdyn pcssatslgt vysdgstyqv ctdtrtneps  
itgtstftqy fsvrestrts gtvvnhfn fwahhgfns dfnyqvave awsgagsav  
tiss

**N**

MLTKNLLLCF AAKAVLAVP HDSVVERSDA LHKLSERSTP SSTGENNGYY YSFWDGGGD  
VTYTNGNAGS YSVEWSNVGN FVGGKGWNP SAKDITYSGN FTPSGNGYLS VYGWTTDPLI  
EYYIVESYGD YNPGSGGTTR GNVSSDGSVY DIYTATRTNA PSIQGTATFS QYWSVRQNK  
VGGTVTTSNH FNAWAKLGMN LGTHNYQILA TEGYQSSGSS SITIQ

**O**

mkvtaasagl lghafaapvp qpvlvsrsag inyqvnyngn ladftydesa gtfsmywedg  
vssdfvvglg wttgssnais ysaeyasgs ssylavygww nypqaeyyiv edygdynpcs  
satslgtvys dgstyqvctd trtnepsitg tstftqyfsv restrtsgtv tvanhfnfwa  
qhgfngsdfn yqvmaveaws gagsasvtis s

**FIGURE 16**

**P**

mrtikffffav aiatvakaqw ggggasagqr ltvngngqtqh kgvadgysye iwldntggsg  
 smtlgsgatf kaewnasyvr gnflarrgld fgsqkkatdy syigldytat yrqtgsasgn  
 srlcvyvgwfq nrgvqgvplv eyyiedwvd wvpdaqgrmv tidgaqykif qmdhtgptin  
 ggsetfkqyf svrqqkrtsg hitvsdhfke wakqggwign lyevalnaeg wqssgiadv  
 kldvyttqkg snpaptstgt vpssaggst angkkftvgn ggnqhkgvnd gfsyeiwlnd  
 tggngsmtlg sgatfkaewn aavnrgnfla rrgldfgsqk katdydyigl dyaatyqta  
 sasgnsrlcv ygvfqnrgln gvplveyyii edwvdwvpda qgkmvtidga qykifqmdht  
 gptinggset fkqyfsvrqg krtsghitvs dhfkewakqg wgignlyeva lnaegwqssg  
 vadvtlldvy ttpkgsspat saaprtrtrt ttrtkslptn ynkcsarita qgykccsdpn  
 cvvyytdedg twgvenndwc gcgveqcassk itsqgykccs dpncvvyfytd ddgkwgvenn  
 dwcgcgcf

**Q**

mvsftsiita avaatgalaa patdvslvar qntpngegth ngcfwswwsd ggaratytn  
 aggsysvswg sgnlvvgkg wnpgtartit ysgtynynqn sylavygwtr nplveyyvve  
 nfgtydpssq sqnkgvtvtsd gssykiaqst rtnqpsidgt rtfqgywsvr qnkrssgsvn  
 mkthfdawas kgmnlqghyy qivategyfs tgnaqitvnc p

**R**

mkrkvkmmaa matsiimaim iilhsipvla griiydnetg thggydyelw kdygntimel  
 ndggtfscqw snignalfrk grkfnsdkty qelgdivvey gcdynpnngns ylcvygwtrn  
 plveyyives wgswrppgat pkgtitqwm gtyeiyyettr vnqpsidgta tfqgywsvrt  
 skrtsgtisv tehfkqwer gmrngkmyev altvegyqss gyanvyknei riganptpap  
 sqspirrdaf siieaeeyns tnsstlqvig tpnngrgigy iengntvtys nidfgsgatg  
 fsatvatevn tsiqirsdsp tgtllgtlyv sstgswntyq tvstniskit gvhdvlfvfs  
 gpvnvndfif srsspvpapg dntrdaysii qaedydssyg pnlqifslpg ggsaigyien  
 gysttyknid fgdgatsvta rvatqnatti qvrlgspsgt llgtiyvgst gsfdtyrdvs  
 atisntagvk divlvfsgpv nvdwfvfsks gt

**S**

mklskikkvl sgtvsalmia saapvvasaa dqqtrgnvvg ydyemwnqng qggasmnpgg  
 gsftcswni enflarmgkn ydsqkknka fgnivltydv eytprgnsym cvygwtrnpl  
 meyyivegw dwpppgndge vkgtvsangn tydirktmry nqpsldgtat fpqywsvrt  
 sqsannqtny mkgtidvthk fdawsaagld msgtlyevsl niegyrsngs anvksvsvtq  
 ggssdngggq qnndwnqgnn nqqqnnndwnn wqggqnnndwn wnnqggqnnnd wnnwggqnnnd  
 wngwnnqggq qnndwnnwgq qnndwnqwnn qggqnnndwn nwgqnnndwn qwnnqggqnn  
 ndwnnwgqgn ndwnqwnnqn nqqgnawngw dnnnnwnqnn qqgnnwdwnn qnnwnnnqgg  
 nndwnqwnnq nnwnnnqggq ndwnqwnnqg qgnndwnqwn nqnnwnqnnn qgnawngwdn  
 nnnwnqwdqn nqwnnqggqn twdwnnqnnw nnnqnnndwn qwnnqggqnn ndwnqwnnqn  
 nnqnnqwdwn nqnnwnqnnn qgnawngwdn nnnwnqwgqg nndwnnqggq ndwnqwnnqg  
 qqgnndwnnq nnwnqggqnn nnsagssdsl kgafskyfki gtsvsheln sgadflkkyh  
 nsitpenelk pesildqgac qqkgnnvntq islsraaql kfceqngial rghtfwysq  
 tpdwffrenf sqngayvskd imnqrlesmi kntfaalksq ypnldvysyd vcnelflnng  
 ggmrgadnsn wvkiygdds fvinafkyarq yapagcklyl ndyneyipak tndiynmamk  
 lkqlgyidgi gmqshlatny pdantyetal kkflstglev qitelditct nsaeqadlye  
 kifklamqns aqipavtiwg tqdtvswrss qnpllfsgy qpkpaydrvm alak

**FIGURE 16**

T

mktfsvtkss vvfamalgma stafaqdfcs naqhsqgkvt itsnqtgkig digyelwden  
 ghggsatfys dgsmdcnitg akdylcragl slgsnktyke lggdmiaefk lvksgaqnv  
 ysyigiygwm egvsgtpsql veyyvidntl andmpgswig nerkgtitvd ggtyivyrnt  
 rtgpaiknsg nvtfyqyfsv rtsprdcgti nisehmrqwe kmgltmgkly eakvlgeagn  
 vngevrqghm dfphakvyvk ngsdpvssss vksssstdap ksssskgngn vsgekackd  
 vmghegketr tggqnnssvt gnvgsppyhy eiwyqgggns mtfydnngtyk aswngtndfl  
 arvqfkydek htyeelgpid ayykwsqqs aggyynyigiy gwtvdplvey yivddwfnkp  
 ganllgqrkg eftvdgdtye iwqntrvqqp sikgtqtfpq yfsvrksars cghiditahm  
 kkweelgmkm gkmyeakvlv eaggsgsgsf vtyfkmtka hplaqpepes ssseakvess  
 sstvalhaap kmelksqnfq vfdmqgrflg tvkldagasv aqvikanfkn agiymvkqgn  
 fmqravavk

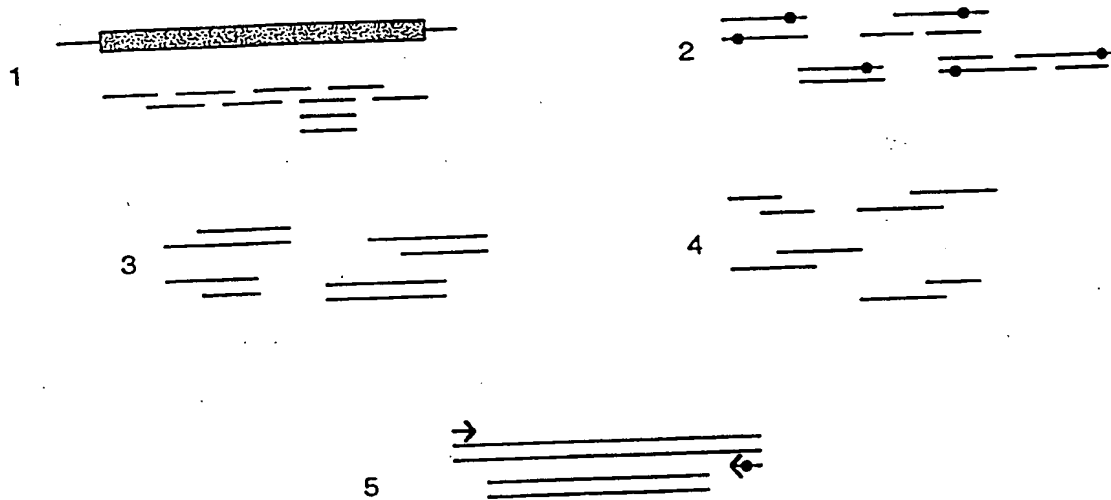
U

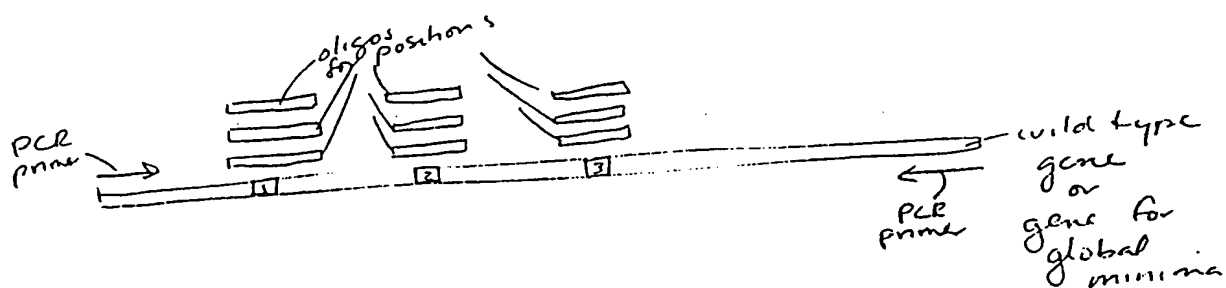
mkvtaafagl lvtafaapap epdlvsrsag inyvnqnyngn lgdfitydesa gtfsmywedg  
 vssdfvvglg wttgssnait ysaeyasgs asylavygwv nypqaeyyiv edygdynpcs  
 satslgtvys dgstyqvctd trtnepsitg tstftqyfsv restrtsgtv tvanhfnfwa  
 hhgfgnsdfn yqvvaveaws gagsasvtis s

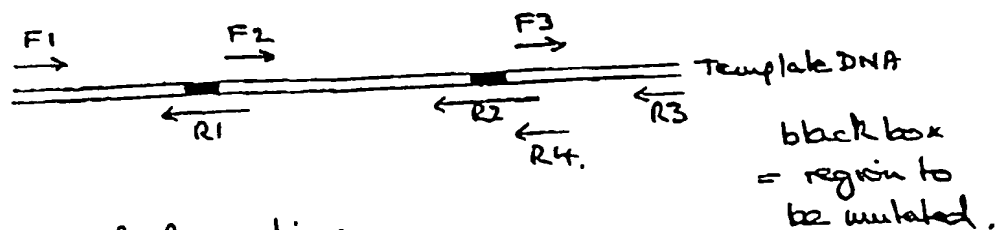
V

mkqvkimflmtmfl  
 gigllffsenaeaaitsneigthdydyef  
 wkdsqsgsgsmtlnsggtfsaqwsnvnnilf  
 rkgkkfdetqthqqignmsinygatynpng  
 nsyltvvgwtvdplvefyivdswgtwrppg  
 gtpkgtinvdggtyqiyettrynqpsikgt  
 atfqqywsvrtskrtsgtisvsehfrwes  
 lgmnmgnmyevaltvegyqssgsanvysnt  
 ltigqsggeqatrveaesmtkggpytsni  
 tspfngvalyangdnvsfnhsftkanssfs  
 lrgasnnsnmarvdlriggqnrgtfyfgdq  
 ypavytinninhgignqlvelivtaddgtw  
 dayldyleir

FIGURE 16

**FIGURE 17**

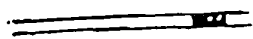
**FIGURE 18**



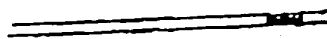
Step 1: Set up 3 PCR reactions:

Products:

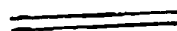
Tube 1:



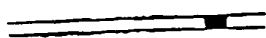
Tube 2:



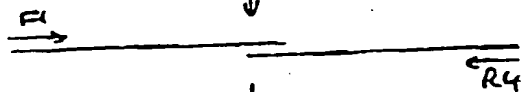
Tube 3:



Step 2: Set up PCR reaction with products of tube 1 + products tube 2 + F1 + R4



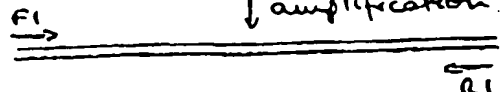
↓ Heat + anneal phase of PCR,



↓ synthesis phase of PCR.



↓ amplification phase, using F1 + R4.



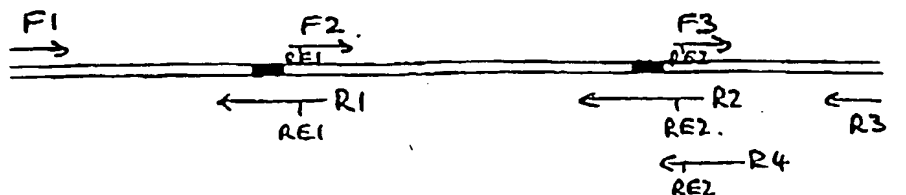
during subsequent cycles.

FIGURE 19A

Step 3.

Repeat step 2 using product from step 2 +  
product from Step 1, tube 3 + primers F1 + R3

**FIGURE 19B**



Step 1 Set up 3 PCR reactions:

Tube 1:

Tube 2:

Tube 3:

Step 2: digest products from Step 1 with suitable restriction endonucleases

Step 3: ligate digested product from Step 2, Tube 2 with digested product from Step 2, Tube 1.



FIGURE 20A



Step 4

Amplify <sup>via PCR</sup> ligated products of Step 3 with F1 + R4.



Step 5. Digest amplified product of step 4 with restriction endonuclease #2.

Step 6

Ligate product from Step 5 with product from Step 2, tube 1.

Step 7

Amplify product from step 6 with F1 + R3

FIGURE 20B

Diagram 3

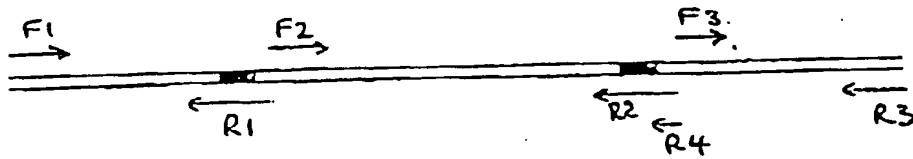


FIGURE 21